FIBRILLATION OF THE AURICLES: ITS EFFECTS UPON THE CIRCULATION.*

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PLATES 43 AND 44.

A little more than two years ago, while working at a peculiar form of heart irregularity exhibited by human subjects, which had been described by Mackenzie[1] as "nodal rhythm," I was able to bring forward considerable evidence[2] to show that the irregularity in question results from fibrillation of the auricles. The evidence for that conclusion was based upon a comparison of curves taken by several methods from the human subject and the experimental heart. The similarity of electrocardiograms taken from patients, and from dogs in which auricular fibrillation had been induced, was noticed almost simultaneously by Rothberger and Winterberg.[3] A fuller analysis of these electrocardiograms supported the hypothesis; and the contention was finally proved by the observation that the oscillations characterizing the clinical electric curves arise from the auricular portion of the heart, and especially by the observation that a similar irregularity occurs in the horse and that this irregularity may be shown by inspection of the heart in that animal to result from auricular fibrillation.[4] Briefly, it has been shown that the commonest form of irregular heart action in man is due to this disturbance of the auricular mechanism. The irregularity is of

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[1] Mackenzie, J., *Quart. Jour. Med.*, 1907–8, i, 39.

[2] Lewis, T., *Heart*, 1910, i, 306; *Brit. Med. Jour.*, 1909, ii, 1528.

[3] Rothberger, C. O., and Winterberg, H., *Wien. klin. Wchnschr.*, 1909, xxii, 839; *Arch. f. d. ges. Physiol.*, 1910, cxxxi, 387.

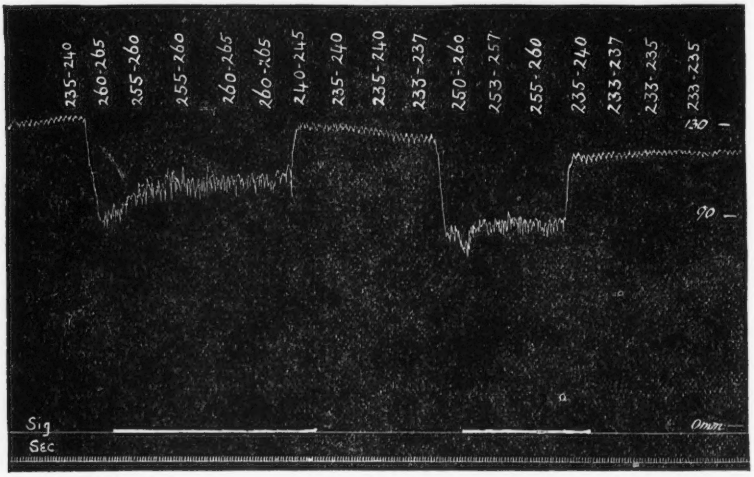
[4] Lewis, T., *Verhandl. d. deutsch. path. Gesellsch.*, 1910, xiv, 112; *Heart*, 1912, iii, 161.

this form in 70 per cent. of all cases of heart failure, as I have been able to demonstrate by electrocardiographic curves taken from over a hundred patients. The conclusion is now generally accepted.

The disturbances of the circulation are so profound in the human subject, when this curious disorder of the heart's action begins, that further study of them seemed desirable. With this object in view, I have undertaken the experiments described in the present communication.

PRELIMINARY OBSERVATIONS UPON METHOD.

The observations have been carried out upon a number of cats and dogs; the former were anesthetized with urethane and ether, the latter with morphia, paraldehyde, and ether. Fibrillation of the auricles was induced by faradic stimulation of the right auricle. In order that the heart should be surrounded by conditions as natural as possible, the stimulation was performed with the chest wall restored, wherever the circumstances of the experiment permitted such restoration. A small window was made in the chest wall over the right auricle, and small flexible electrodes of the fish hook type were fastened into



TEXT-FIG. 1. Cat 30. A kymographic curve, showing the effects on arterial and venous blood pressures of faradization of the right auricle. The actual readings of the venous manometer are written on the curve. A subtraction of 174 mm. must be made to arrive at the actual venous pressure in mm. of magnesium sulphate (specific gravity, 1,046).

TABLE I.

Arterial Pressure.

The Effect of Auricular Fibrillation in Cats. The Figures Represent the Actual Rises and Falls.

Cat.	Onset.	After first few seconds.	After second few seconds.	Offset.	Remarks.
28	−24	+10	+10	0	
	−36	+16	0	+20[5]	
29	0	0	0	0	
	−10	0	+ 4	+ 6	
30	+20	− 8	0	−12	Rate during fibrillation 102 only.
	−25	+ 8	+ 8	+16	
	−24	+10	0	+ 8	
32	0	0	0	0	
	−20	+18	0	+ 8	
31	− 2	0	0	+ 2	Heart exposed.
	−12	+ 6		+ 6	
	−16	+12		+ 4	
41	+12	0	0	−12	Rate during fibrillation 128 only.
	−44	0	0	+38	
	−30	+15	+15	0	
	−32	0	0	+30	
42	−12	+18	0	+ 6	Heart exposed.
	− 2	+14	+ 4	− 4	
	+10	0	0	−10	Rate during fibrillation 193 only.
43	−48	+28	+12	+ 8	Heart exposed.
	−36	0	0	+20	
44	−18	+ 8	0	+10	Heart exposed.
	−30	+20	+ 2	+ 8	
45	−28	+14	+10	+10	Heart exposed.
	−34	+20		+18	
46	+10	0	0	−10	Rate rise of 55 beats per minute. Heart exposed.
	−30	0	0	+30	
	−40	0	0	+40	
47	− 6	0	0	+ 6	Heart exposed.
	−42	+40		0	
	−12	+ 8		+ 6	
48	−16	0	0	+10	Heart exposed.
49	−10	0	+ 4	+10	
50	−14	0	0	+10	Heart exposed.
51	−18	+10	0	+ 6	Heart exposed.
	−20	0	0	+20	
52	−40	+10	+ 2	+24	Heart exposed.
53	−28	+ 8	+ 2	+14	Heart exposed.

[5] The pressure fell 36 mm. at the onset, rose again 16 mm., ran horizontally, and rose 20 mm. at the offset. Thus it returned to its original level.

the auricular substance. The electrode wires were rubber coated and passed between the layers of the chest wall. Any air which had entered the pleura was withdrawn, the window in the chest wall being closed.

CHANGES IN MEAN ARTERIAL PRESSURE.

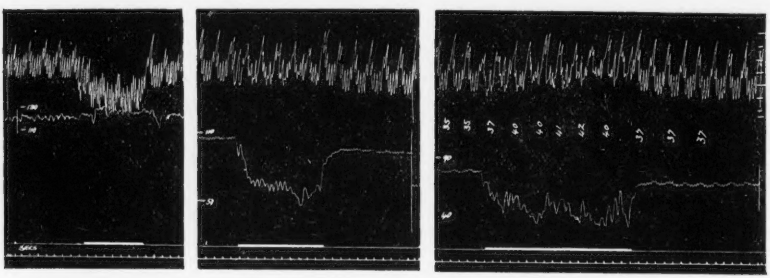
The following observations are based upon manometric readings in five dogs and thirty-seven cats.

TABLE II.

Arterial Pressure.

The Effect of Auricular Fibrillation in Dogs.

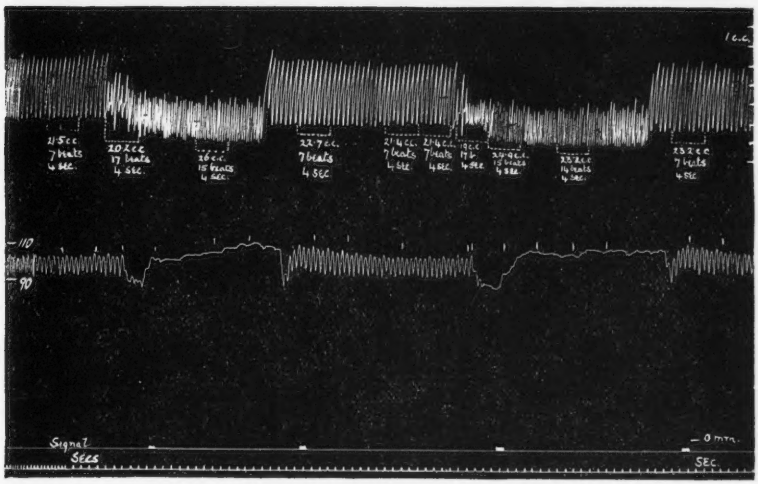
Dog.	Onset.	After first few seconds.	After second few seconds.	Offset.
CP	+ 9	0	0	−10
CL	−30	0	+8	+20
	−40	+12	0	+12
CK	−44	+34		0
	−82	+36	0	+30
CO	+ 9	0	0	− 9
	−15	+21	−6	0
CN	−30	+16	0	+12
	−28	+22	0	0



a *b* *c*

TEXT-FIG. 2, *a*, *b*, and *c*. Three portions of a tracing showing arterial pressure and heart volume curves taken from a series of observations. The first curve (figure 2, *a*) shows a rise of mean arterial pressure at the onset of fibrillation, an increase of cardiac output and a decrease of cardiac volume. The second curve (figure 2, *b*), in which the blood pressure has fallen, shows a deep fall of arterial pressure, a fall of cardiac output, and a slight and progressive increase of cardiac volume. In the third curve (figure 2, *c*), in which the arterial pressure has fallen still lower, a fall of blood pressure occurs at the onset of fibrillation and it is accompanied by a decrease of cardiac output, and by a considerable and progressive increase of cardiac volume. With the rise of pressure in figure 2, *a*, the heart action is far more regular than with the falls of blood pressure in figure 2, *b* and *c*.

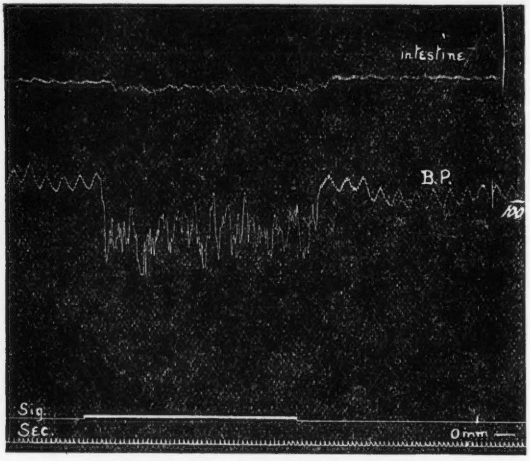
The effect of auricular fibrillation, and its accompanying phenomena, is very varied in experiment. At its onset, the mean arterial pressure may remain unchanged; it may rise at once (text-figure 2, *a*); but much more commonly there is an immediate fall (text-figure 1). The greatest rise has been noted in a cat, and amounted to twenty millimeters of mercury; the greatest fall occurred in a dog, and amounted to eighty-two millimeters of mercury. The fall which is customary in both cats and dogs is usually twenty to forty millimeters of mercury. When an immediate



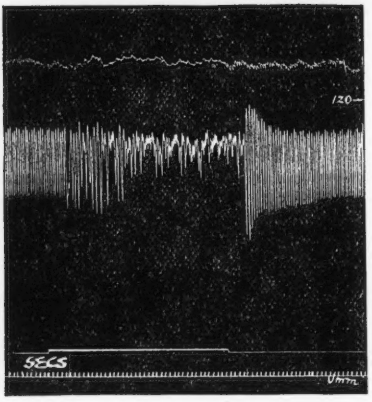
TEXT-FIG. 3. Cat 42. Curves of arterial pressure and heart volume, showing the effects of auricular fibrillation. The output and number of heart beats per four seconds are marked from time to time along the upper or cardiometer curve. The output varies in the same direction as the blood pressure. In the cardiometer curve, systole is indicated by a downstroke. Note the diminution of heart volume during the period of fibrillation.

rise occurs, it may be succeeded by a fall of less degree within a few seconds; when an immediate fall occurs, it is usually succeeded by a rise of less degree within a few seconds. Thus the circulation tends to accommodate itself to the altered conditions and this is especially so when there is no initial fall of

pressure. In most experiments where there is a fall of pressure,—and these are in the majority,—a quick return towards the previously recorded pressure is observed, as I have stated; the original



a



b

TEXT-FIG. 4, *a* and *b*. Figure 4, *a* (cat 33), gives arterial pressure and intestinal volume curve, showing the effects of faradization of the right auricle. Figure 4, *b* (dog CP), gives curves of arterial pressure and intestinal volume, showing the effects of faradization of the auricle. During the period of fibrillation the mean blood pressure is raised and the intestinal volume is increased.

pressure may be regained or surpassed (text-figure 3); commonly the rise becomes slower and a distinct shoulder is present in the curve (text-figures 1 and 3), which then runs more or less horizontally or rises gradually. The commonest type of arterial curve consequently consists of an abrupt fall, a preliminary and steep rise of about a third or half the extent of the former, and finally a horizontal or a gradually rising curve, the latter spread over a few seconds or a minute. Eventually the pressure lies within five to fifteen millimeters of the original pressure, and when, or shortly after stimulation of the auricle ceases, the pressure returns to normal. This return is abrupt, and often goes beyond the normal limit (text-figure 4, *a*), returning again within a few seconds. If the fibrillation is maintained for five minutes or more, the arterial pressure has usually risen to within five or fifteen millimeters of the original level, though it may remain lower.

Tables I and II give selected figures of the arterial pressure changes in the eighteen cats and five dogs.

CHANGES IN VENOUS PRESSURE.

The venous pressures have been taken from the femoral vein by means of a manometer containing magnesium sulphate in solution (specific gravity, 1,046). The tip of the venous cannula was passed into the femoral vein so that its mouth lay in the iliac vein; and the readings were taken by noting the position of the meniscus in relation to a millimeter scale, and were recorded in writing on the curve at intervals of a few seconds. All experiments in which no movements of the meniscus with respiration were discerned have been discarded.

The changes of venous pressure when the auricle passes into fibrillation are slight. They have been determined in a number of observations upon five cats and two dogs, and the observations permit certain general statements to be made. When the arterial pressure is maintained at the original level, the venous pressure also remains unchanged. A rise of venous pressure is the customary event, for there is usually a fall in arterial tension; and the latter being usually twenty to forty millimeters of mercury, the former generally amounts to five to ten millimeters of magnesium sulphate solution. The arterial and venous pressures move in opposite directions, and this relation is generally maintained not only during the preliminary, but also in the later stage of an observation. Thus,

if the arterial fall is deep and the recovery prompt, the venous pressure after first rising falls again; and when at the offset of fibrillation the arterial pressure reaches its original level, or when it is restored during the fibrillation period, the venous pressure also falls to normal. While these general rules hold good, there are natural exceptions from time to time, for the fluctuations of venous pressure as a result of the change in cardiac mechanism are usually small, and the venous pressure shows variations which are independent of the nature of the heart beat (tables III, IV, and V).

TABLE III.

CAT 47.

Arterial pressure in mm. of mercury.	Venous pressure in mm. of magnesium sulphate.	
82	72–75	
80	72–75	
		Auricles fibrillated.
52	82–92	
52	82–87	
74	77	
80	72	
80	72	
		Normal rhythm restored.
80	72	
78	72	
78	72	
78	70–72	
78	70–72	
		Auricles fibrillated.
60	82	
56	87	
70	92	
78	77	
76	75	
76	75	
75	75	
76	72	
76	77	
		Normal rhythm restored.
78	74	
78	60–72	
78	60–72	
79	60–72	

TABLE IV.

CAT 30.

Arterial pressure in mm. of mercury.	Venous pressure in mm. of magnesium sulphate.	
132	91	
	81–90	
	81–90	
120	80–86	
		Auricles fibrillated.
98	103–104	
	91–96	
	88–96	
104	88–96	
		Normal rhythm restored.
111	71–81	
104	74–81	
		Auricles fibrillated.
81	94–101	
	86–92	
92	82–91	
		Normal rhythm restored.
96	70–76	
98	71–79	
		Auricles fibrillated.
78	88–98	
	86–94	
	85–94	
	88–96	
	86–95	
	90–96	
94	83–92	
		Normal rhythm restored.
111	71–79	
116	71–79	
120	71–78	
		Auricles fibrillated.
84	94–101	
99	86–91	
	84–91	
	91–98	
	91–98	
102	91–96	
	81–91	
		Normal rhythm restored.
120	70–78	
	70–76	
		Auricles fibrillated.
132	61–69	

TABLE IV (*Continued*).

CAT 30.

Arterial pressure in mm. of mercury.	Venous pressure in mm. of magnesium sulphate.	
90	86–91	
104	81–86	
	81–86	
	86–91	
108	86–91	
		Normal rhythm restored.
125	66–71	
	61–66	
	61–66	
123	59–63	
		Auricles fibrillated.
84	76–81	
	79–83	
90	81–86	
		Normal rhythm restored.
118	61–66	
	59–63	
	59–61	
118	59–61	

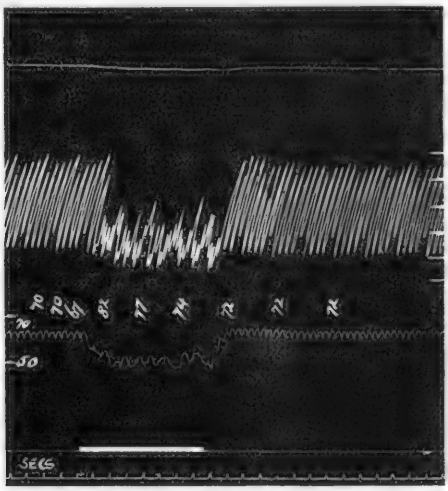
TABLE V.

CAT 29.

Arterial pressure in mm. of mercury.	Venous pressure in mm. of magnesium sulphate.	
116	67	
	69	
		Auricles fibrillated.
118	70	
	75	
114	77	
		Normal rhythm restored.
110	80	
100	40	
		Auricles fibrillated.
104	45	
	47	
102	50	
		Normal rhythm restored.
96	50	

TABLE V (*Continued*).

Arterial pressure in mm of mercury.	Venous pressure in mm. of magnesium sulphate.	
128	43	
	42	
124	39	
		Auricles fibrillated.
118	45	
118	45	
116	45	
		Normal rhythm restored.
118	37	
114	37	
78	33	
78	33	
		Auricles fibrillated.
	CAT 29.	
68	40	
68	32	
68	32	
		Normal rhythm restored.
78	33	
76	33	



TEXT-FIG. 5. Cat 47. A record of arterial and venous pressures and of heart and intestinal volumes, showing the effects of auricular fibrillation. At the time this curve was taken the intestinal record was not working well and the dip in the curve, indicating diminution of intestinal volume, is only just seen.

That the changes in the arterial and venous systems are the direct outcome of altered cardiac mechanism is indicated by the general manner in which the pressures behave. The peripheral circulation is affected passively. This is also shown by records of intestinal volume. Examples of venous readings are given in text-figures 1, 2, *c*, and 5.

CHANGES IN THE INTESTINAL CIRCULATION.

The volume changes in the small intestine were measured in the usual manner by enclosing a loop of the gut in an air-tight box connected to a recorder. The activity of the vasomotor system was checked at the termination of each series of observations by the injection of a suitable dose of adrenalin solution, so as to demonstrate the customary fall of volume with the rise of blood pressure. Observations have been made upon six cats and two dogs from this point of view; they have given uniform results.

The volume curves at the onset of fibrillation have always run parallel with the arterial pressure changes; a fall of arterial pressure is accompanied by a fall of volume (text-figure 4, *a*), and conversely a rise of blood pressure, be it primary or secondary, is accompanied by a rise of intestinal volume (text-figure 4, *b*). The changes in volume at the offset of fibrillation correspond in the same fashion with the changes of arterial pressure.

As might be anticipated, alterations such as occur in the arterial and venous pressures are consequently explained by the altered cardiac mechanism and by this alone. Observations which have been made upon the output of the heart are confirmatory of this conclusion.

CHANGES IN THE VOLUME OF THE HEART.

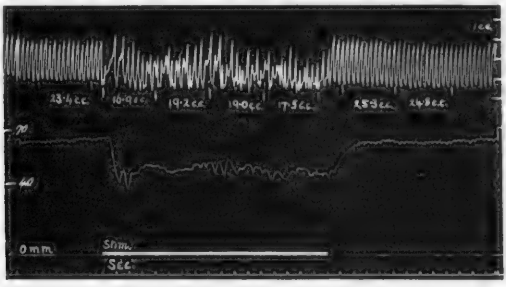
The mean volume changes of the heart at the onset and offset of fibrillation of the auricles have been estimated by opening the chest, cutting away all but a short sleeve of pericardium, enclosing the whole heart in a glass cardiometer and rendering the mouth of the cardiometer air-tight by tying the sleeve of pericardium firmly over it. The records were taken in twelve cats by means of the recorder designed by Starling[6] for the purpose. The recorder may be calibrated and the excursions are uniform for like changes of volume within the necessary limits.

The volume of output has been calculated by adding together the length of all the downstrokes, representing systoles, over a definite period of time. The

[6] Jerusalem, E., and Starling, E. H., *Jour. Physiol.*, 1910, xl, 286.

excursions yielded by changes of volume of 1 cm. are ascertained at the subsequent calibration. The method gives a fairly accurate expression of output in cubic centimeters while the heart beats regularly, and its accuracy is independent of the variations in excursion consequent upon respiration. It is also, I believe, fairly accurate when the ventricle beats irregularly, though under such circumstances the readings tend to be too high. This is due probably in some part to the inefficiency of certain ventricular beats. While the auricles are fibrillating, a large number of ventricular contractions fail to open the aortic valves; yet it is impossible to avoid a certain excursion in the recording lever as a result of change in the shape of the heart when no blood leaves it, even with the closest fitting cardiometer. Such excursions add to the sum of the calculated output. Consequently in some of the experiments the readings during the fibrillation period have been obviously too high, but the comparison of readings over periods of change of blood pressure has given uniform results. The figures obtained have been regarded relatively rather than absolutely, and treated in this fashion they are of value.

When the blood pressure changes in response to onset or offset of fibrillation, or when it changes during the fibrillation, the output also changes. If there is a fall of pressure there is a fall of output; if there is a rise of pressure there is a rise of output. Examples are given in tables VI and IX and text-figures 2, 3, 5, and 6.



TEXT-FIG. 6. Cat 43. Vagi cut. Arterial pressure and heart volume curves, showing the effects of auricular fibrillation. The heart output and arterial pressure vary in the same directions. There is only a slight decrease in the mean size of the heart.

Mean Volume Changes.—When the auricles pass into fibrillation, the heart demonstrates a change in mean volume, of which the direction is practically constant. The volume decreases.[7] This is

[7] I shall refer briefly to certain exceptions to this rule in my concluding remarks.

TABLE VI.

Cat.	Before fibrillation.			After onset.				A few seconds later.			After offset.		
	Blood pressure (mean).	Heart rate.	Output per ¾ sec. in c.c.	Blood pressure (mean).	Heart rate.[8]	Output per 4 sec. in c.c.	Volume change in c.c.	Blood pressure (mean).	Heart rate.[8]	Output per 4 sec. in c.c.	Blood pressure (mean).	Heart rate.	Output per 4 sec. in c.c.
42	98	105	21.5	87	255	20.2	−1.4	108	225	26.0	100	105	22.7
	98	105	21.4	87	255	19.0	−1.5	104	210	24.9	104	105	23.2
	Left vagus cut.												
	104	172	20.9	82	270	17.1	−0.3	100	262	21.7	98	150	21.7
43	82	135	24.8	60	165	14.8	−1.3	60	165	15.0	84	135	26.2
	Right vagus cut.												
	68	180	19.8	46	300	15.0	0	47	292	15.0	67	180	23.0
	Left vagus cut.												
	65	172	23.4	46	255	16.9	−0.4	50	270	19.0	64	180	24.8
44	82	169	29.4	63	285	16.0	−1.2	62	277	16.0	82	177	28.0
	73	170	15.3	34	258	6.1	+1.0	64	255	11.8	75	168	17.2
47	82	202	40.7	57	365	30.3	−1.4	76	250	39.1	78	200	39.8
	78	195	38.7	58	360	22.7	−1.4	75	250	37.7	78	200	40.6
49	67	186	25.5	58	300	18.0	−0.4				66	177	25.2[9]
	All cardiac nerves divided.												
	60	163	22.4	49	270	15.2	0	55	285	19.4	63	157	23.4

[8] Heart rates are calculated from cardiometer curves; approximate readings.

[9] Fibrillation lasted only a short while.

indeed obvious from mere inspection of the organ. The decrease in size is seen whether the arterial pressure rises (text-figures 2, *a*, and 3), falls (text-figures 5 and 6), or remains stationary. It is due entirely to change in the size of the ventricles. The auricles, it is true, are included in the cardiometer, but they pass into a position of diastole, or increased mean volume, when fibrillation begins. Examples of the mean volume change will be found in tables VI and IX, and in the figures to which reference has been made.

SUMMARY OF PREVIOUS OBSERVATIONS.

As far as I have recorded my observations, they show that fibrillation produces changes in arterial pressure, which consist of certain falls and certain rises of pressure. The venous pressure moves in the opposite direction from the arterial pressure. If we compare increase and decrease of arterial pressure with increase and decrease of intestinal volume, it may be said that they change in the same direction, as does also the cardiac output. We may conclude, therefore, that the changes in arterial pressure are due solely to the change in the mechanism of the heart beat and we may seek the cause of all the circulatory pressure changes in this organ.[10]

We have to consider the cause of the usual steep fall of arterial pressure at the onset of fibrillation. We have to determine the cause of the partial or complete recovery in certain instances, the cause of the variation in the reaction in different experiments, and the cause of the fluctuation of pressure during fibrillation in a given experiment. We have also to seek the reason for the change of cardiac volume.

It has become more and more apparent, during the course of the experiments, that the explanation of most of these phenomena lies in the acceleration of the heart's rate, though it has not been altogether easy of proof. The effects of auricular fibrillation and of simple tachycardias, provoked by applying regular induction shocks to the right auricle, have been compared.

[10] The changes are not affected by section of the vagi or sympathetics.

DEGREE OF VENTRICULAR ACCELERATION WHEN THE AURICLES FIBRILLATE, AND THE EFFECT OF REGULAR ACCELERATION OF SIMILAR EXTENT.

A number of figures, giving the ventricular rates before and after the onset of fibrillation, will be found in tables VI and IX; these rates were calculated from cardiometer curves. In blood pressure tracings the rate of the ventricle cannot often be estimated during the period of fibrillation, because the irregularity of the ventricle is so gross and because usually so many beats of the ventricle fail to affect the arterial pressure. The curves of heart volume give more accurate readings, and readings which are approximately correct. But at the present time, that the true grade of acceleration may be appreciated, I give the following figures (table VII), taken from electrocardiographic curves in a separate series of experiments. The conditions of the animals in these experiments were similar to those described in the present series.

TABLE VII.

	Before onset of fibrillation.	After onset of fibrillation.
Dogs	95	185
	170	230
	157	237
	183	330
	149	255
	102	205
	117	198
	165	240
	160	270
	185	283
Cats	151	304
	192	330

Other figures are given in the sequel.

The acceleration of the ventricle, when the auricles pass into fibrillation, is from 50 to 100 per cent. under normal experimental conditions. The rate of the ventricle during the period of fibrillation varies in dogs and cats approximately between 180 to 330 beats per minute.

In several experiments, a simple manometric comparison was made between the effects of auricular fibrillation and regular ac-

celeration. The mercurial manometer was employed, and after electrodes had been fastened to the right auricle the chest was closed. Examples of the results are seen in table VIII. The

TABLE VIII.

	Ventricular rate rises from	Blood pressure changes from
	Cat 52.	
Fibrillation	207 to ——	107 to 86
Tachycardia	184 to 190	102 to 104
Tachycardia	176 to 285	113 to 109
Tachycardia	176 to 288	113 to 107
Tachycardia	178 to 242	112 to 112
Tachycardia	178 to 236	113 to 115
Tachycardia	177 to 208	111 to 113
Tachycardia	176 to 210	112 to 113
Tachycardia	176 to 236	112 to 113
Tachycardia	175 to 256	112 to 110
Tachycardia	180 to 286	112 to 103
Tachycardia	174 to 256	110 to 110
Tachycardia	176 to 258	110 to 108
Tachycardia	171 to 268	111 to 103
Tachycardia	174 to 276	109 to 93
Tachycardia	172 to 310	107 to 73
Fibrillation	174 to ——	109 to 79
	Cat 53.	
Tachycardia	236 to 395	90 to 51
Tachycardia	228 to 364	89 to 66
Fibrillation	230 to ——	88 to 62
Tachycardia	186 to 338	77 to 43
Tachycardia	188 to 305	77 to 47
Tachycardia	200 to 258	80 to 74
Tachycardia	195 to 246	81 to 78
Tachycardia	190 to 236	82 to 81
Tachycardia	183 to 236	82 to 81
Tachycardia	192 to 236	83 to 81
Tachycardia	192 to 204	82 to 82

ventricular rate during the periods of simple tachycardia could be estimated readily from the arterial curves and signal of stimulation. The estimation of rate during the periods of fibrillation was not possible. It will be seen that considerable acceleration of the ventricle (to 242, 256) may occur without change of arterial pressure, or

that such increase of rate may be accompanied by a slight rise or slight fall of arterial pressure. It will also be seen that, as higher rates are reached, small falls of pressure ensue and that eventually, as the rate increases, the fall becomes greater, until when the heart rate rises to 300 or more per minute, falls result which are equivalent to those obtained in fibrillation. These rates are of the degree known to occur when the auricles are fibrillating. The actual figures naturally vary very much from experiment to experiment, and an absolute comparison is by no means easy. It is difficult to obtain comparable falls with simple and with irregular acceleration, for the degree of fall varies with so many factors; for example, with the original height of venous and arterial blood pressure and with the original heart rate. The chief defect of the method now described lies in the impossibility on most occasions of ascertaining the ventricular rate during the period of fibrillation. Other examples, from experiments in which the chest was open, and in which the rates were calculated from cardiometer curves, are given in table IX. These figures confirm the conclusion that the rate is

TABLE IX.

Vagi and Sympathetics Cut.

Cat 50.

	Ventricular rate rises from	Blood pressure falls from	Heart decreases in size by approximately	Output per 4 secs. in c.c. decreased from
Fibrillation	200 to 283[11]	86 to 72	1.3 c.c.	
Fibrillation	192 to 275[11]	84 to 72	1.3 c.c.	
Fibrillation	174 to 230[11]	80 to 66	1.4 c.c.	25.2 to 21.0
Tachycardia	170 to 190	71 to 71	0.25 c.c.	22.6 to 21.2
Tachycardia	172 to 301	73 to 73	0.33 c.c.	23.9 to 22.8
Tachycardia	181 to 204	60 to 59	0.25 c.c.	18.7 to 14.9
Tachycardia	190 to 263	55 to 49	0.5 c.c.	17.8 to 10.8

Cat 51.

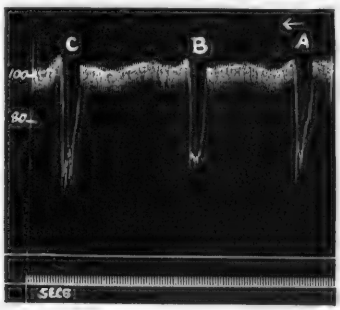
Sympathetics Cut.

Fibrillation	156 to 205[11]	58 to 48	1.4 c.c.	23.0 to 21.2
Tachycardia	155 to 195	58 to 57	1.0 c.c.	
Tachycardia	153 to 185	57 to 56	0.8 c.c.	24.2 to 23.1
Tachycardia	150 to 223	59 to 54	1.4 c.c.	23.3 to 17.5
Tachycardia	150 to 190	60 to 59	1.0 c.c.	
Fibrillation	140 to 216[11]	58 to 47	1.0 c.c.	
Fibrillation	139 to 196[11]	56 to 42	1.0 c.c.	

[11] These figures, taken from counts in the cardiometer curve, are approximate.

a predominant factor in producing the usual fall of blood pressure, though they indicate that acceleration, when the ventricular action is regular, is less profound in its effects than is acceleration when the ventricular regularity is disturbed. The last named experiments were performed for the sake of heart volume curves, rather than for the rate readings.

The question has been approached in a more direct manner in further experiments. In these, animals in which the chest wall was subsequently closed have been utilized. In two experiments the blood pressures were recorded with the mercurial manometer, and the rates were calculated from simultaneous electrocardiograms. While the blood pressure was running steadily, a faradic current or repeated induction shocks at varying rates were passed into the auricle through the chest wall by means of the specially protected electrodes. When a pressure fall of uniform extent was obtained after several faradic stimulations, a fall of similar amount, as far as this was possible, was obtained with regular induction shocks; this was accomplished by varying their rate of incidence. The experiment was then repeated, and at the same time electrocardiograms



TEXT-FIG. 7. Cat 67. Unlike the remainder of the figures, this blood pressure curve reads from right to left. It shows a fall of blood pressure obtained with (*A*) faradization of the auricle, (*B*) regular acceleration of the auricle, and (*C*) faradization of the auricle. The heart rates corresponding with these three curves were 216, 225, and 231 respectively.

were taken from the right fore limb and left hind limb. Such experiments are not easy, because, apart from variations in the general

line of blood pressure, similar curves of blood pressure during fibrillation are not often obtainable; and it is necessary that the comparison should be made in the shortest possible time, so that the two curves lie close together. Text-figure 7 is an example of such an experiment, and selected figures are given in table X. The read-

TABLE X.

		Blood pressure falls from	Heart rate rises from	
I	Fibrillation	108 to 94 = 14	188 to 216	
	Tachycardia	112 to 96 = 16	185 to 249	
II	Fibrillation	120 to 100 = 20	180 to 220	
	Tachycardia	126 to 90 = 36	179 to 261	
	Tachycardia	112 to 87 = 25	180 to 249	
III	Fibrillation	86 to 68 = 18	150 to 207	
	Tachycardia	104 to 85 = 19	154 to 222	
IV	Fibrillation	102 to 68 = 34	145 to 216	(Figure 7, *A*, *B*, and *C*.)
	Tachycardia	100 to 65 = 35	147 to 225	
	Fibrillation	100 to 62 = 38	147 to 231	

ings confirm the previous observations. Regular and irregular accelerations of similar rate produce approximately equal falls of pressure, but the irregular acceleration is almost always a little more profound in its effects. That is to say, if equal falls of pressure are obtained, it is subsequently found that the rate during the period of regular acceleration slightly exceeds that of the period of fibrillation.

When the auricles fibrillate, a number of heart beats fail to affect the arterial curve and are entirely lost. The series of experiments was therefore extended, and arterial curves were taken from five animals with Hürthle's manometer, the two records, arterial and electrocardiographic, lying on the same plate. Repeated observations are required before curves are secured in which systolic and diastolic readings are alike and in which a comparison is valid. Nevertheless, out of a large number of such curves, some may be chosen from which the required data are obtainable; the readings conform to those obtained in other ways. Two observations are chosen for illustrative purposes; in selecting them attention has been paid to the form of the arterial curve during the period of fibrilla-

tion. In figure 1, *a* and *b* (plate 43), there are two falls of blood pressure. The original systolic and diastolic pressures are the same in both curves during the interval of depression; the average diastolic pressure is somewhat lower in the curve which shows fibrillation. The average ventricular rate is 300 for the fibrillation curve, and 306 for the curve which shows regular acceleration. Thus the falls of pressure for similar rates are approximately equal, though again the fibrillation has a somewhat more profound effect. Figure 2, *a* and *b* (plate 43), are from a similar experiment. The original pressures in each curve are almost the same. The initial fall is somewhat greater in the fibrillation curve; eventually the blood pressure rises above that shown at a similar phase of figure 2, *a*, and once more falls below it. The respective rates for figure 2, *a* and *b*, are 315 and 328.

If we continue to consider the cause of the usual fall of arterial pressure, the part played by the average rate of heart beat at such times seems clear. It appears to be the all important factor. There is, however, another conceivable cause of the fall of pressure. It has been recently emphasized by Gesell[12] as a reason for a pressure fall in auricular fibrillation. Gesell produced complete heart-block experimentally; thus he dealt with hearts in which the ventricles beat at uniform rates; he then experimented with the auricles, eliminating their systoles by inducing fibrillation in them. In this way he produced falls of blood pressure of 10 mm. of mercury. His conclusion, from this and other experiments, that the auricles aid in ventricular filling to an extent which may not be neglected, cannot be questioned; but at the same time, the absence of auricular filling cannot be considered a serious factor in the production of the falls of pressure with which we now deal. Such a possibility requires discussion, because in the comparison which has been instituted in the present series of experiments this factor is neglected. In those instances in which regular acceleration is induced and in which it is of a sufficiently high grade to produce large falls of blood pressure, auricular filling does not occur. The auricles are as much out of action as they are when they fibrillate, for each auricular contraction falls with the preceding ventricular systole; this is evident in the electrocardiograms; *P* falls with the preceding *T* (figure 2, *a*). I draw attention especially to this fact, because the comparison between the regular and irregular acceleration is more perfect. The question arises as to what extent the absence of auricular filling influences the result. Gesell produced falls of pressure of only small extent, about 10 mm. of mercury. It must be remembered that in these experiments each ventricular cycle was accompanied by four or five auricular cycles at the time when fibrillation of the auricles was induced. The ventricle fills, under ordinary circumstances, chiefly during the early and late phases of diastole. The information which we require is the relation of

[12] Gesell, R. A., *Am. Jour. Physiol.*, 1911, xxix, 32.

the amount of early filling by static pressure to the late or forcible auricular filling. When the ventricle beats slowly, the filling during mid-diastole is slow, unless, as in Gesell's experiments, there is additional or forced feeding as a result of supernumerary auricular contractions. Ten mm. of mercury, therefore, are probably too much to allow as the amount of the fall which is attributable to the auricular factor. That the auricular factor is an unimportant one is clearly evidenced by the varying effect which fibrillation of the auricles has upon arterial pressure. In place of a fall, the blood pressure may remain unaltered; it may actually rise. If in figure 3, *a* (plate 44), the beats marked by asterisks are compared, the second is seen to succeed an auricular contraction. The two beats are of almost the same values; the diastolic pressures are equal, the systolic pressure of the second beat exceeds that of the first by less than 7 mm. of mercury. The difference is fully accounted for by the difference in lengths of preceding pauses; these are 8.6 and 9.4 thirtieths respectively. The filling which precedes the two contractions seems to have been equally rapid. The absence of auricular filling when the auricles fibrillate seems to be almost if not quite compensated for by increased venous pressure, when there is a fall of arterial pressure.

The degree of acceleration not only accounts, I believe, almost entirely for the usual steep fall, but accounts completely for the variations in the amount of fall from time to time, and also for the fluctuations of pressure which are seen during the course of a single observation. Comparisons are of most value under the last named circumstance. When the ventricle beats its fastest, a number of contractions produce no arterial pulsation. The shortest cycles of figure 3, *a* and *b*, correspond to the steepest falls of pressure and to those portions of the curve at which no arterial pulsations are visible. After a careful examination of the curves in my possession I find that there is no exception to the rule which these figures illustrate. If previous events are taken into consideration, each rise and each fall of pressure are fully accounted for by the lengths of the cycles found to correspond to it. That the curve of rate of fall or of rise should not be absolutely parallel to the curve of lengths of pauses is natural, for the effect of a longer or shorter pause is not confined to the immediately succeeding cycle.[13] This is especially the case where the initial fall and terminal rise are concerned; for over these periods the supply of blood to the heart does

[13] Where there is an abortive contraction, as in figure 3, *b* (marked with asterisks), the beat which follows has a greater effect on arterial pressure than may be accounted for by the previous pause; this evidently results from some of the blood of the previous diastole being carried over for one heart cycle.

not immediately meet the requirements of the particular pauses in question; the supply may be excessive, or it may be scanty.

A short pause is equivalent to a fleeting acceleration; a long pause is equivalent to a fleeting retardation. Moreover, a short pause counts more heavily than a relatively long one; the study of regular acceleration teaches that great acceleration produces effects out of proportion to the increase of rate. If over two equal periods the rate is the same, but over one the heart action is regular while over the other long and short pauses are mixed, the effect is more profound over the latter. Whenever the blood pressure rises at the onset of fibrillation, or wherever it rises above the original point during the progress of fibrillation, the action of the ventricle is more regular, both in the incidence of the excursions and the amplitude of the beats, in arterial and cardiometer curves (text-figures 2, *a*, and 3); it is also slower. The rates corresponding to the four rises of pressure tabulated in table I were 102, 128, 193, and 255.[14] Where the ventricle beats fast, the arterial pulse and cardiometer curves are very irregular (text-figures 5 and 6), for many of the contractions of the ventricle fail to raise the aortic valves. The initial falls of pressure in text-figure 3 are marked in the cardiometer curve by very irregular heart action; the upstrokes of diastole are notched by the weak contractions which are abortive. The periods of raised pressure in the same figure correspond to more regular heart action. Contrast with this the gross irregularity of text-figure 6; it is maintained throughout, and the pressure is low throughout. When it is remembered that such gross irregularity signifies rapid action, the reason of the fall and its maintenance is more obvious. The same point is illustrated by the falls of text-figure 2, *b* and *c*. Where the pulse irregularity is great, and beats are dropped, the pressure falls steeply; where the action is more regular the pressure rises; and where beats are dropped, as we have seen, the pauses which precede them are curtailed.

Thus the fluctuations of arterial pressure are explained by a detailed consideration of heart rate. Falls are due to excessive rapidity, rises to less rapid action; a rise above the initial pressure is comparable to the occasional small rises which result from simple acceleration. Briefly, the changes in the peripheral circulation are

[14] In this instance the original rate was 200.

most serious when the acceleration is greatest. That is a conclusion which is fully borne out by clinical experience. When fibrillation is present, the rate of ventricular action is one of the most important indications of the gravity of the condition as a whole.

The initial fall is usually the deepest, as I have stated, though this is not invariably the case; but it is deepest in a sufficient percentage of cases to place its occurrence beyond the possibility of coincidence. The most rapid ventricular action is also found at the onset of the disturbance and accounts for it. The reason why the ventricle beats more rapidly when fibrillation begins is probably that at the onset of fibrillation the tissues which transmit the impulse from auricle to ventricle are usually unfatigued. When after a fall the initial pressure is recovered and fibrillation persists, the recovery may be assigned to lessened conduction power between auricles and ventricles. The recovery is probably due also in a measure to increased venous pressure. The falls which accompany acceleration are the result of curtailment of the diastolic periods; a heightened venous pressure, by promoting the filling, will tend to compensate for it.

The final rise at the termination of the fibrillation needs no detailed explanation; it is due, as is the frequent overriding of the initial pressure, to the transference of the obstructed blood from the venous to the arterial system.

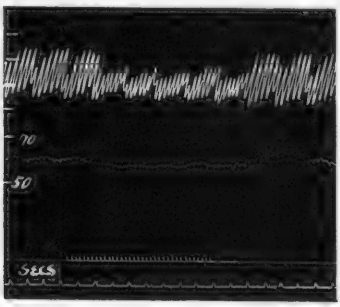
CAUSE OF THE MEAN VOLUME CHANGE.

It has been stated that when the auricles pass into fibrillation, the heart volume decreases. A similar reaction is found when the heart rate shows regular acceleration (table IX and text-figure 8). Up to a certain point the greater the acceleration, the greater is the decrease of volume. It may be, as in table IX, cat 51, that if regular and irregular accelerations are approximately equal in degree, the volume decrease is of approximately equal amounts. On the other hand, and perhaps more commonly, the regular acceleration may be accompanied by a relatively smaller decrease (table IX, cat 50).

The chief factor in producing diminution of heart capacity when the auricles fibrillate is the same as that which produces it when the acceleration is regular. The diastolic intervals are curtailed; it is the diastolic volume which shows the chief reaction; the systolic volume also suffers, though to a less extent. The heart is smaller at all phases of its beat. But the decrease in heart volume is independent of the velocity of flow through it. The flow is usually decreased when there is a fall of pressure, but it may be increased when there is a rise above the initial pressure (text-figure 3). Thus, although the diastolic intervals are decreased, the blood flow into the heart may be even in excess of the initial flow. Under these circumstances the average rate of inflow must have increased. The reason for such an increase is not to be found in an increased venous pressure; it must consequently be due to a lowering of mean diastolic ventricular pressure. There seems, therefore, to be no alteration of the ventricular function which is spoken of as "tone." The decrease of heart volume appears to result

from more purely mechanical causes; shortening of the diastolic periods as a result of acceleration is sufficient alone to account for it. Section of all the cardiac nerves does not influence the reaction.

The decrease of mean heart volume is the almost constant reaction of the normal heart; but in the human subject the onset of auricular fibrillation is often accompanied by obvious increase of volume. I have seen dilatation on a few occasions in the present series of experiments. The actual cause of this unusual reaction has been difficult to ascertain, but it may have been due, as probably is



TEXT-FIG. 8. Cat 50. Arterial pressure and heart volume curves, showing the effects of stimulating the auricle with a succession of regular induction shocks. The blood pressure is lowered and the heart volume and output are decreased.

the reaction of the human heart, to nutritional changes in the ventricular muscle. A weak or failing muscle will not necessarily react in the same manner as does fresh and vigorous tissue.

There is perhaps no quality of heart muscle which it is more difficult to estimate at a given moment than its state of exhaustion, and I write with hesitancy of the relation of dilatation to such a quality at the onset of fibrillation. More observations are required before any views can be exposed with confidence. Where it occurs, the dilatation of the ventricles at the onset of fibrillation is usually seen towards the end of an experiment. It is usually associated with relatively low blood pressure. This will be evident from an examination of table VI; dilatation of the heart or unchanged volume accompany initially low arterial tensions.

On one occasion a series of curves was obtained from an animal, in which a complete transition was seen from decreasing to increasing volume. Three curves from this series are shown in text-figure 2. The blood pressure, which falls steadily throughout the whole series, rises in the first curve when fibrillation begins; it falls in the second and third curves. The volume decreases by several cubic centimeters in the first curve; it shows slight progressive increase in the second, and considerable and progressive increase in the third. The explanation of this series of volume changes seems to be that from first to last there was a steady exhaustion of the muscle, so that while at first, being vigorous, it reacted in the usual fashion, maintaining its tone, toward the end the increase of rate was speedily followed by loss of tone.

SUMMARY AND CONCLUSIONS.

When the auricles fibrillate, the following effects are observed.

1. The arterial blood pressure may rise, fall, or remain stationary. Usually it falls. If it falls, it generally rises again towards or to the initial pressure.

2. The venous pressure changes are the reverse of the arterial.

3. The intestinal volume and the cardiac output changes are in the same direction as those of arterial blood pressure.

4. From these observations it may be concluded that the peripheral circulatory effects are purely passive.

5. The volume of the heart decreases except in instances where there is reason to believe that the circulation is failing.

All the changes described in the foregoing paragraphs, and also the variations in blood pressure reactions which occur from time to time, are attributable to alterations in the rate of ventricular contraction. Similar, though perhaps less profound changes, are seen when the heart rate accelerates in like degree in response to regular induction shocks.[15]

EXPLANATION OF PLATES.

PLATE 43.

FIG. 1, *a* and *b*. Cat 64. Hürthle manometer curves from the carotid, and electrocardiograms. In *a* the auricle was faradized. In *b* regular induction shocks were employed. The falls of pressure are equal; the respective rates of ventricular action are 300 and 305. The time marker is in thirtieths of a second.

FIG. 2, *a* and *b*. Cat 65. Similar curves from another animal; *a* = stimulation with regular induction shocks at a rate of 328 per minute; *b* = faradization; rate of ventricle 315 per minute. The falls of pressure are almost equal.

PLATE 44.

FIG. 3, *a* and *b*. Cat 67. Two electrocardiograms and two Hürthle curves; *a* shows fluctuations of arterial pressure and the return to the normal rhythm at the very end of the curve. The fluctuations of pressure were due to variations in heart rate. The measurements of the beats are expressed in thirtieths of a second. *b* shows the recovery of arterial pressure towards the end of a period of fibrillation, and its cause,—decrease of heart rate.

[15] This conclusion applies only to induction shocks applied to the auricle; the effect of similar stimulation of the ventricle is far more profound. Stimulation of the auricle seems to result in contractions of the ventricle, which are the most efficacious.

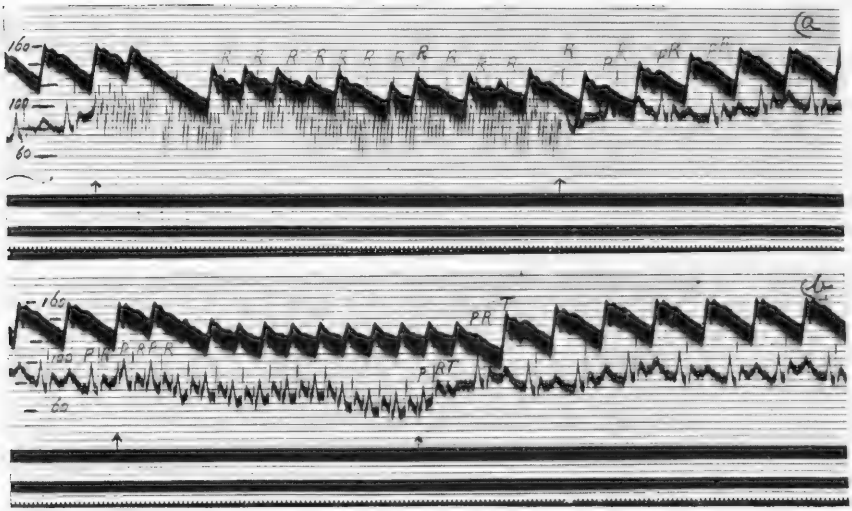


FIG. 1

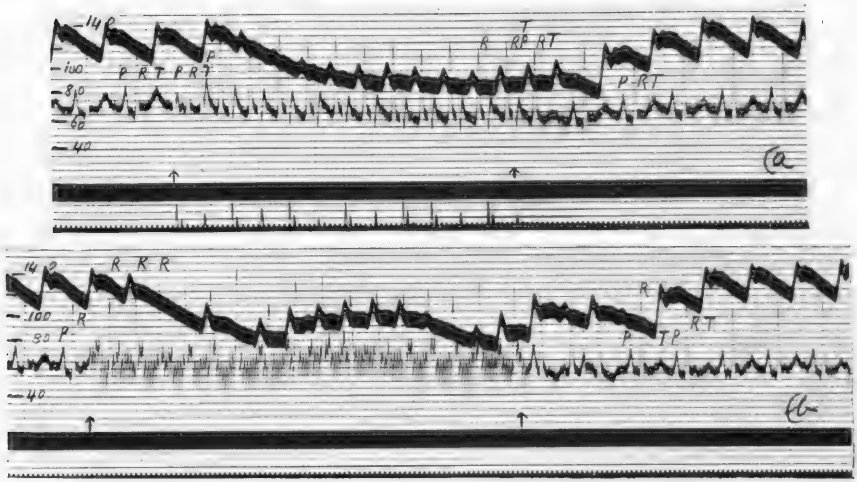


FIG. 2.

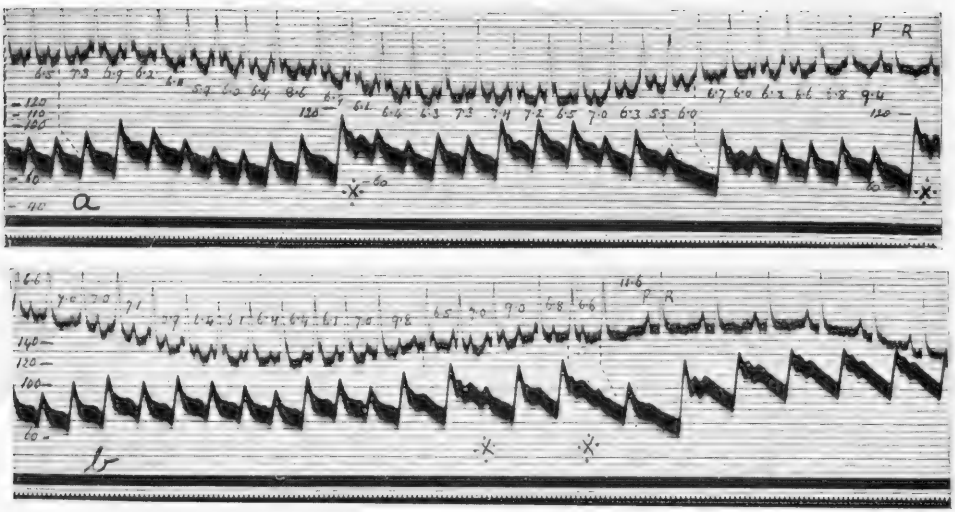


FIG. 3.

STUDIES UPON THE CHARACTERISTICS OF DIFFERENT CULTURE MEDIA AND THEIR INFLUENCE UPON THE GROWTH OF TISSUE OUTSIDE OF THE ORGANISM.*

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PLATES 45–54.

In a previous article[1] I have called attention to the fact that heat exerts a marked influence upon serum when used as a culture medium. This was observed in cultures in serum agar of the bone marrow of adult guinea pigs. It was supposed that the influence which the heating of the serum exerted upon its value as a culture medium and upon the growth of tissue in it was indicated truly by the rapidity of the emigration of ameboid cells from the piece of tissue. I did not know then whether these cells really multiplied, or whether they only migrated. In order to reach conclusive results it was necessary to carry these experiments further.

The experiments, the results of which I shall give in this paper, were planned to investigate thoroughly the influence of heat upon serum as a culture medium, taking into consideration at the same time the value of different media and the conditions necessary for the growth *in vitro* of adult and embryonic tissue.

To distinguish between emigration and multiplication of cells in a culture may be very difficult. *In vitro* the lymphoid cells show ameboid movements, but motility is not at all limited to these cells, for it may be manifested by every connective tissue cell, even the highly differentiated cells of the cornea. The movements of the connective tissue cells, however, are very slow compared with the rapid movements of the ameboid lymphoid cells, which under the

* Received for publication, June 3, 1912.

[1] Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xv, 397.

microscope may be seen to change from second to second. Since the first stage of growth is very often only an emigration, it is not permissible to conclude that all the cells that are seen surrounding the original fragment represent a growth of the latter.

Direct observation under the microscope of cells that are dividing is proof, of course, that they multiply, but to make these observations great patience is required, and even with patience one may fail to see cells that are dividing. Another evidence of growth is the occurrence of mitotic figures in emigrated cells that divide indirectly. But for cells that divide amitotically, such as leucocytes, this criterion cannot be used. Mitotic figures found in the original piece itself by making sections of it, as Oppel[2] did, do not prove that there is growth outside of the organism, for these figures might, of course, be present before the fragment was removed and transplanted to the plasma.

Real growth *in vitro* cannot be acknowledged as proved unless a new tissue that grows from the periphery forms around the original fragment.

This is most easily observed in cultures of certain epithelial organs (hypophysis, thyroid), which form *in vitro* strands, columns, and arches of cells that lie close together, the basal cells of which are inactive and show but little alteration, while the peripheral cells change and multiply continually. The same observation may be made in growing connective tissue (endothelial cells) (figure 13), but here it is not so marked nor so easily confirmed, because the connective tissue cells have motility and are not so liable to keep close together or to form sheets of tissues.

Such a newly formed and growing tissue proves beyond doubt that multiplication of cells has occurred, and the mitotic figures present in this tissue afford additional evidence of growth.

In these experiments I have cultivated adult and embryonic tissues in different media, that is, in autogenic and homogenic plasma, homogenic serum (heated and unheated) plus 2 per cent. agar, homogenic serum, Ringer's solution plus 2 per cent. agar, and in Ringer's solution alone.

[2] Oppel, A., *Anat. Anz.*, 1912, xl, 464.

The plasma cultures were prepared in the ordinary way described by Carrel and Burrows.[3]

The cultures in serum and agar and Ringer's solution plus agar were prepared according to the technique described by me in another article.[4]

Cultivation of tissue in serum and in Ringer's solution was carried out in drops hanging from the under surface of cover-slips that were sealed to hollow slides, care being taken that the amount of fluid surrounding each piece of tissue covered it in a very thin film.

Adult Tissue.—From several adult cats, rabbits, and guinea pigs, I cultivated spleen, bone marrow, hypophysis, and thyroid gland. Real growth, that is formation of a new tissue around the original fragment was obtained in practically all experiments when the tissues were cultivated in plasma. Morphologically the organs behaved almost exactly alike whether taken from cats, rabbits, or guinea pigs.

From thyroid and hypophysis there appeared on the second and third days solid columns of epithelial cells that represented in all probability the specific glandular cells of these organs (figures 16 and 24); in some cases long spindle connective tissue cells (figure 15) grew out and the presence of these cells apparently prevented the development of the epithelial structures.

In the first stages of growth of spleen and bone marrow there are always emigrations of ameboid cells, which start moving one or two hours after the beginning of the incubation of the culture. On the third day the ordinary connective tissue cells begin to grow and, after incubation for five or six days, form a new connective tissue that surrounds the original fragment.

While in plasma all the organs of the adult animals examined give rise to real growth, usually of their specific cells, but sometimes only of the connective tissue cells, in not one case have I found real growth of the organs of adult animals when cultivated in the other media mentioned; that is, in Ringer's solution, in Ringer's solution plus 2 per cent. agar, in serum, and in serum plus 2 per cent. agar.

[3] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

[4] Ingebrigsten, R., *loc. cit.*

In these media the emigration of cells is very often seen, and from lymphatic tissue (bone marrow) in serum plus agar this emigration is very rapid. But the formation of new tissues has not been observed.

Embryonic Tissue.—It is quite different, however, with embryonic tissue which grows well in plasma, but just as well and even better sometimes in some of the other media.

Tissues from chick and cat embryo were examined. The cat fetus was almost at full term when taken from the uterus of the mother. The chick embryos used were fifteen to sixteen days old.

Peritoneum, skin, liver, and thyroid of the fetal cat grew very well in serum agar. They started to grow on the first day and formed around the original fragment during the following two or three days an extensive area of new tissue (epithelial cells from the thyroid, liver, and skin, and connective tissue cells from the peritoneum) (figures 13, 14, 17, 18, 21, and 22). In serum alone these tissues grow very little, a few connective tissue cells only spreading along the cover-glass.

In Ringer's solution and in Ringer's solution plus agar they did not grow at all.

Heart, spleen, skin, and liver were cultivated from the chick embryos fifteen to sixteen days old. These organs grew very well in serum plus agar.

The epithelial cells of the liver and skin grew much more extensively and vigorously in chick serum plus agar than in chick plasma (figures 17 and 18). In serum agar they grew usually in one layer between the serum agar clot and the cover-glass. From the heart and spleen of chick embryos connective tissue cells developed actively in serum agar (figures 1 to 12), but not as well as in plasma. After three to five days the spleen cultivated in plasma showed a wider growth of elongated connective tissue cells, and the area of emigrated cells was larger and denser in the plasma than in the serum agar (figures 11 and 12). Chick heart in plasma gave rise to a dense framework of connective tissue that surrounded the fragment evenly. In serum plus agar the same kind of cells developed but on a smaller scale.

In serum, connective tissue from chick heart grew rather exten-

sively along the cover-glass in a thin layer, but it did not grow so well as in serum agar. In serum, chick liver and spleen showed an emigration of cells, but no growth.

In Ringer's solution and in Ringer's solution plus agar some slight emigration of cells occurred from chick spleen, but these cells lost their ameboid movements within the first twenty-four hours, while the same kind of cells, emigrated from spleen in serum, showed ameboid movements for at least three days (figures 7 and 8). In Ringer's solution and in Ringer's solution plus agar, no growth of the cells from the sixteen day chick embryo has been observed.

I wish to emphasize in this place the differences found between adult and embryonic tissues. The former does not grow at all in any medium but plasma, while embryonic tissue develops vigorously also in serum plus agar.

Furthermore, in Ringer's solution and in Ringer's solution plus agar, I found no growth of the cells from the fifteen to sixteen day old chick embryos.

Even within the embryonic stage there are apparently great differences in the energy of proliferation of cells of different ages. The energy of growth *in vitro* decreases as the age of the embryo increases, and the culture experiments that succeed when performed with a chick embryo that is eight days old may be completely unsuccessful with a chick embryo sixteen days old.

Thus, in an extensive series of experiments, Lewis and Lewis[5] have cultivated several tissues of chick embryo in various salt solutions. They generally used chick embryos from seven to nine days old, and in some cases embryos that were thirteen to fifteen days old. In their experiments they observed emigration of cells as well as formation of syncytial membranes. Many of their illustrations are rather convincing as to the presence of multiplication among the cells. In a few experiments in which I cultivated in Ringer's solution connective tissue from chick embryos that were six to eight days old, I had corresponding pictures.

Lewis and Lewis, however, express it as their conviction that the

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

salt solutions utilized by them were protective and not nutrient in action, and this view is certainly correct.

Of the cultures that they prepared, the percentage that was positive decreased as the age of the embryos utilized increased. This varying percentage shows that the very young embryonic tissue has such a tremendous energy of proliferation and is so little dependent on the constituents of the media in which the cells live that they must be considered as constituting a peculiar sort of tissue. The limits within which these salt solutions of Lewis may be varied quantitatively and qualitatively are so extensive, that they cannot be supposed to be of great importance in the metabolism of these cells. By their tremendous energy of proliferation they grow perhaps in spite of the media instead of because of them, and, as Lewis and Lewis themselves say, they grow within the limits determined by the amount of food stored up in the body of each individual cell.

They have, therefore, a comparatively short existence in salt solution, while in nutrient media they live much longer and, as shown by Carrel,[6] may even be kept in permanent life. There is, therefore, a fundamental difference between the life of very young embryonic tissue in salt solution (a preservative medium) and in plasma (a nutrient medium). If we wish to realize the relationship between these two media, we may compare the growth in salt solution with the pale and whitish buds that grow out of potatoes in the spring, if they are placed in a dark and damp cellar, while the growth in plasma and serum agar resembles the powerful, young green leaves that sprout out above the surface of the soil when the potato is permitted to draw energy and nutrition from light, air, and ground. The life and growth of these young embryonic cells in salt solution can hardly be called a culture in the true sense of this word.

Influence of Heat. Homogenic Serum.—In order to ascertain whether the influence of heat exerted upon serum is of importance, not only for the emigration and survival of ameboid cells but also for the multiplication and the growth of tissue cells, it was necessary to cultivate embryonic tissue in heated and unheated serum, because adult tissues do not grow at all in sera.

[6] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516.

Spleen, liver, and skin of chick and cat embryos have been cultivated in heated and unheated homogenic serum plus agar. Spleen of the fetal cat gave only a small growth in serum agar, and was, therefore, not suitable for my purpose. Spleen of a sixteen day chick embryo, on the other hand, grew very well in serum agar and was well adapted for comparative experiments. I found that the growth in unheated serum agar was quantitatively different from that in heated serum plus agar. In the former there appeared after two and three days several elongated cells that formed around the fragment a "corona radiata" that was very similar to that in plasma, while these cells did not develop in heated serum plus agar (figures 10 and 11). In the latter the area of emigrated cells was smaller than in unheated serum plus agar. In these experiments the chick serum had been heated to 52° to 53° C. only. Chick serum cannot be heated beyond that temperature without the occurrence of a precipitation with a subsequent deficit of proteins, and probably salts, in the fluid. The maintenance of the percentage of proteins was, of course, necessary.

The cultivation of epithelial tissues from cat and chick embryo in heated and unheated homogenic serum agar has not given the results that might be expected from the results obtained with chick spleen. In many of the experiments, liver and skin grew better in unheated than in heated serum agar, but in some cases they have grown as well, and in a few cases even slightly better in heated than in unheated serum (cat serum was heated to 58° to 59° C. for half an hour and chick serum heated to 52° C. for half an hour).

The epithelial cells of liver and skin grew very quickly and extensively in one or two layers between the nutrient clot and the coverglass. The epithelial cells were possibly more dependent upon mere mechanical and physical conditions, as, for example, the space available between the clot and the glass, and the degree of adhesion between these two structures, than were the connective tissue cells, which grew in many different layers through all the surrounding medium and therefore responded more quickly than the epithelial cells to changes in the medium, whatever these might be.

This possibly explains also why embryonic epithelial cells grow better in serum agar than in plasma; for the epithelial cells have a

tendency to spread rapidly along a smooth surface and find better conditions between the serum agar clot and the cover-glass than between the plasma clot and the glass, since the plasma adheres very closely to the glass.

Heterogenic Serum.—In agar plus serum from cats, dogs, and rabbits, I have cultivated in these experiments the heart of a sixteen day old chick embryo.

In the plasma from cats, dogs, and rabbits, a piece of chick heart gave rise to the growth of a connective tissue framework that surrounded the fragment evenly but not densely. In serum agar from these same animals a piece of chick heart grew somewhat differently, a thin layer of spindle connective tissue cells spreading from the fragment as a sheet of tissue or growing out in small bushes or bundles.

A comparison of the quantitative growth can easily be made. I found when comparing the growth of chick heart in unheated and heated heterogenic sera plus agar that heated heterogenic serum is a better culture medium than unheated. On the third day all the cultures examined showed more extensive growth in heated serum plus agar than in unheated serum, and the cells of the former were in a better condition.

Sera of dogs, cats, and rabbits are all hemolytic for the red blood corpuscles of chickens, and it is to be expected that the improvement as a culture medium, obtained by heating the serum, is due to the destruction of its hemolytic powers. Whether this is the whole explanation or whether there are other factors to be considered (specific cytolysins), further experiments will perhaps reveal.

In several series of experiments I have determined the relative hemolytic power of heterogenic sera against chicken and guinea pig blood cells, and then, by preparing cultures from the tissues of the animal that supplied the blood cells in the hemolysis experiments (chicken and guinea pig), I have tried to determine whether there is an inverse ratio between the hemolytic power of a heterogenic serum and the rate of growth in it. Plasma was also used.

Chick heart cultivated in agar plus serum from cats, dogs, and rabbits, after the relative hemolytic powers of these sera for

chick red cells had been determined to be 7.7:7.1:2.0, showed that the areas of growth, measured by the micrometer on the third day in the three sera had the relationship of 3:5:12. The same inverse ratio between the hemolytic powers of heterogenic serum and the growth in it was found when guinea pig spleen and bone marrow were cultivated in agar plus serum of rabbits, dogs, and cats. The limits of the growth in these cases were not determinable with exactness, and I am, therefore, unable to give figures.

In heterogenic plasma, on the other hand, this inverse ratio between the hemolytic power and the extent of the growth in it was not constant. The growth of chick heart in cat plasma, for instance, is better than in dog plasma, even if the relative hemolytic power of cat serum and dog serum for chick blood cells is indicated by 7.7:7.1.

This seems to indicate that the character of the fibrin framework in the plasma clot is of some importance for the growth of cells in it, and that perhaps the structure of the fibrin framework is able to modify the growth even as much as chemical and biological differences are able to.

I have, therefore, started a study of the fibrin framework in different plasmas. The results of these studies are not yet conclusive, but I wish to state that small differences between the characters of the framework of plasma from different species are present. A detailed report of these investigations will be given in another article.

CONCLUSIONS.

1. There is a great difference between embryonic and adult tissue as far as their growth outside of the organism is concerned. Adult tissue grows only in plasma. Embryonic tissue grows also very well in serum and serum plus agar. In Ringer's solution and in Ringer's solution plus agar no growth occurs, whether embryonic or adult tissue is employed; survival and emigration of cells are seen to some extent.

2. For the growth of connective tissue cells of chick embryo, unheated homogenic serum is a better culture medium than heated serum. The growth of epithelial cells is not thus influenced.

3. Heated heterogenic serum is a better culture medium for growth of embryonic connective tissue cells than unheated.

4. There is an inverse ratio between the hemolytic power of heterogenic sera and the extent of growth of tissue in them. This inverse ratio is not found in heterogenic plasmas.

EXPLANATION OF PLATES.

PLATE 45.

Camera lucida drawings made on the third day of incubation of cultures in different media of the spleen of a sixteen day chick embryo. The drawings show the relative extent of the emigration of cells and the growth of connective tissue cells. Growth of these cells occurred only in figures 5 and 6 and is indicated in these figures by the *dark* grey area surrounding the black central spot (the original piece of tissue). The *light* grey area represents the emigrated cells.

FIG. 1. Chick spleen in Ringer's solution.
FIG. 2. Chick spleen in Ringer's solution plus 2 per cent. agar.
FIG. 3. Chick spleen in serum.
FIG. 4. Chick spleen in heated serum plus 2 per cent. agar.
FIG. 5. Chick spleen in unheated serum plus 2 per cent. agar.
FIG. 6. Chick spleen in plasma.

PLATE 46.

Photograph of cells that had emigrated and grown out of pieces of spleen of a sixteen day old chick embryo. The pieces had been incubated in different media for three days. The relative extent of the growth is shown in figures 1 to 6.

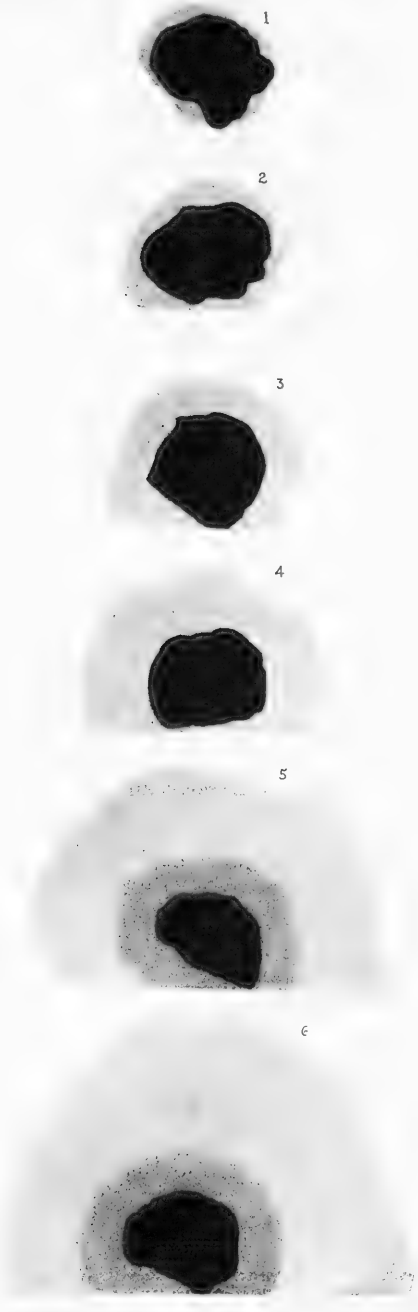
FIG. 7. Chick spleen in Ringer's solution.
FIG. 8. Chick spleen in Ringer's solution plus agar.
FIG. 9. Chick spleen in serum.
FIG. 10. Chick spleen in heated serum plus 2 per cent. agar.
FIG. 11. Chick spleen in unheated serum plus 2 per cent. agar.
FIG. 12. Chick spleen in plasma.

Notice the presence of elongated cells in figures 11 and 12 only. Further, note that ameboid movements are still present on the third day in serum (figure 9), while they stopped within twenty-four hours in Ringer's solution and in Ringer's solution plus agar (figures 7 and 8). Figures 7 to 9 are from photographs of the fresh unstained cells. Figures 10 to 12 are from cells stained with hematoxylin.

PLATE 47.

FIG. 13. A three day culture of peritoneum of fetal cat, in agar plus unheated serum.

FIG. 14. A high power magnification of one of the cells in mitotic division.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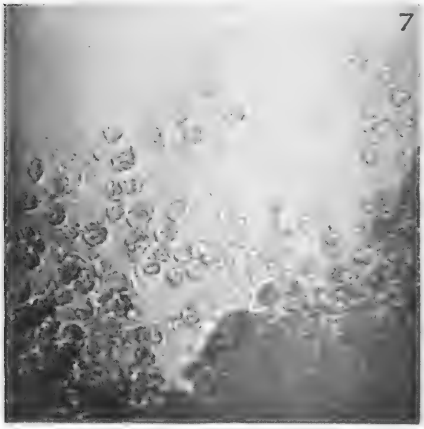
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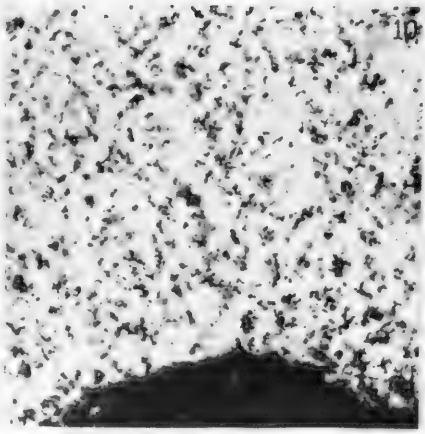
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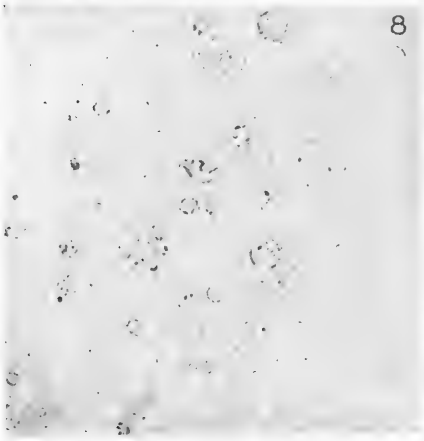
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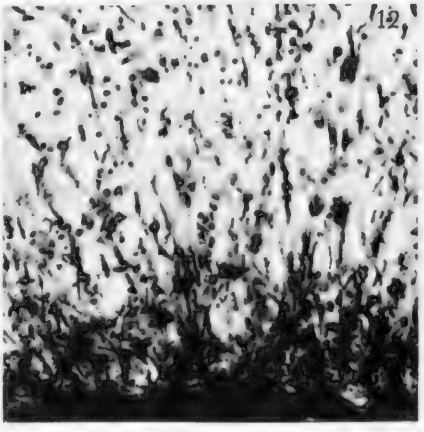
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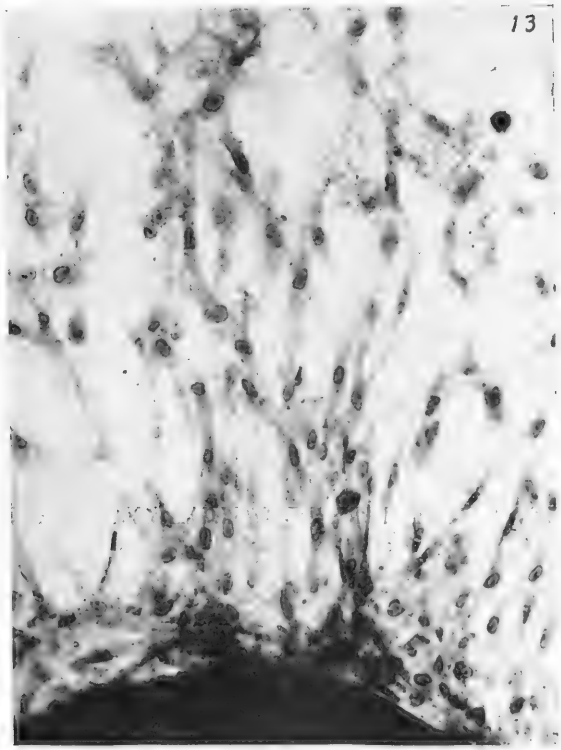
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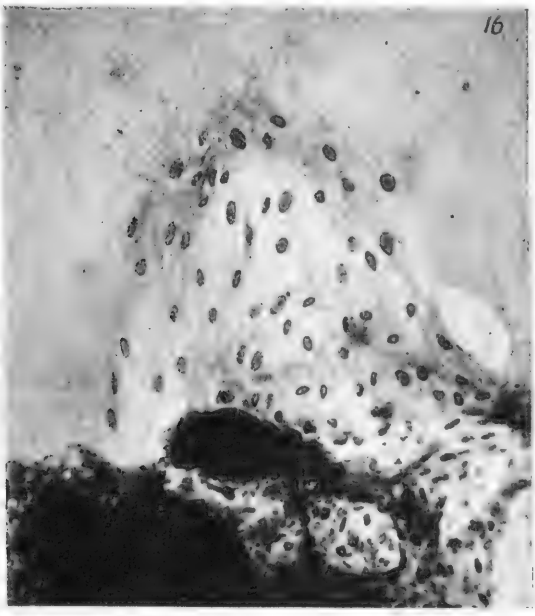
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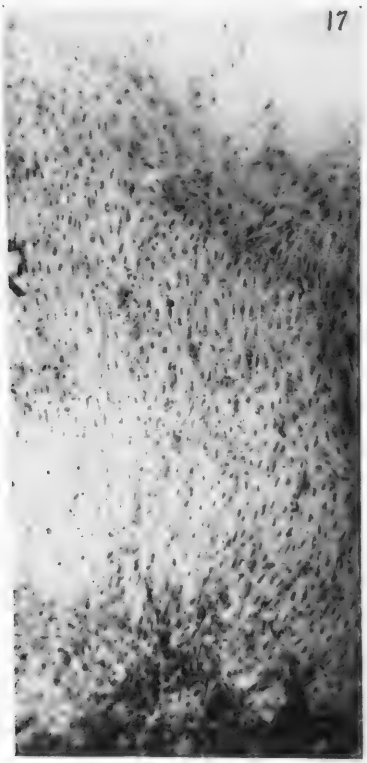
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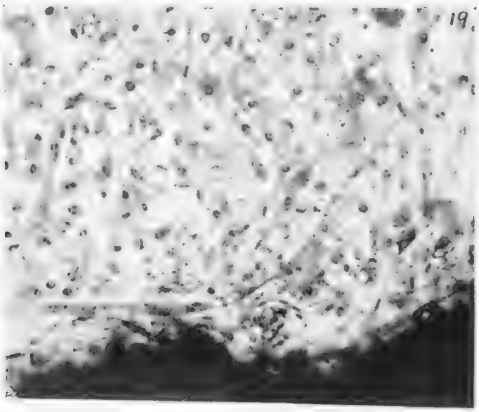
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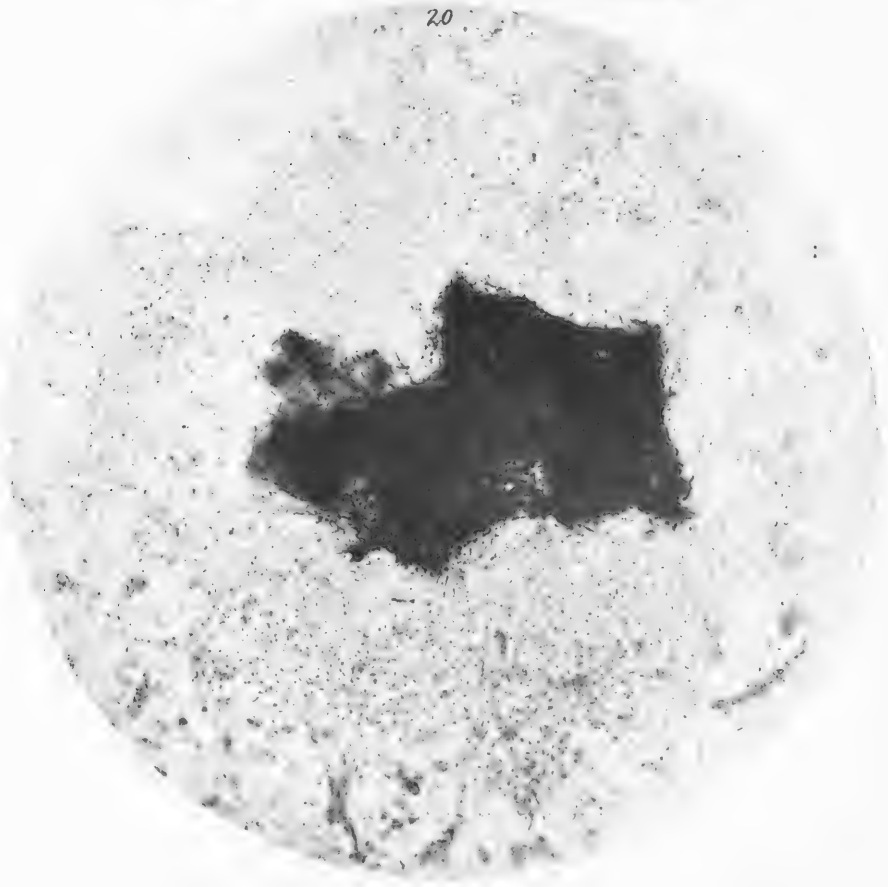
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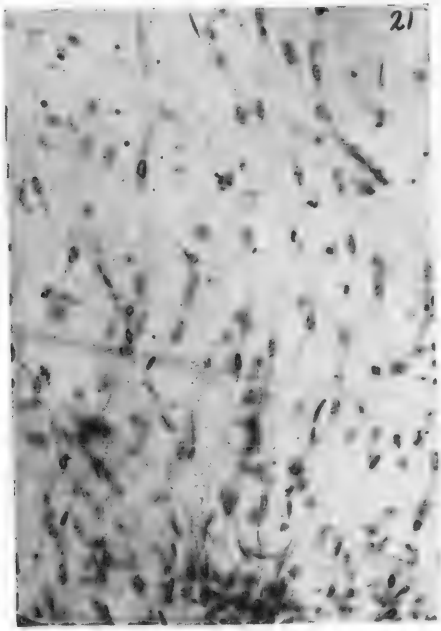
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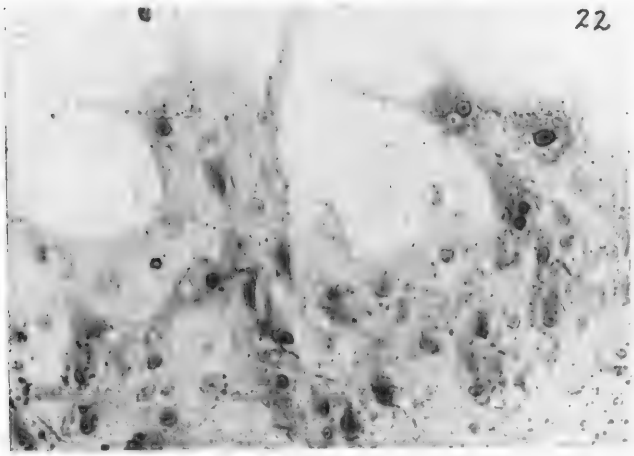
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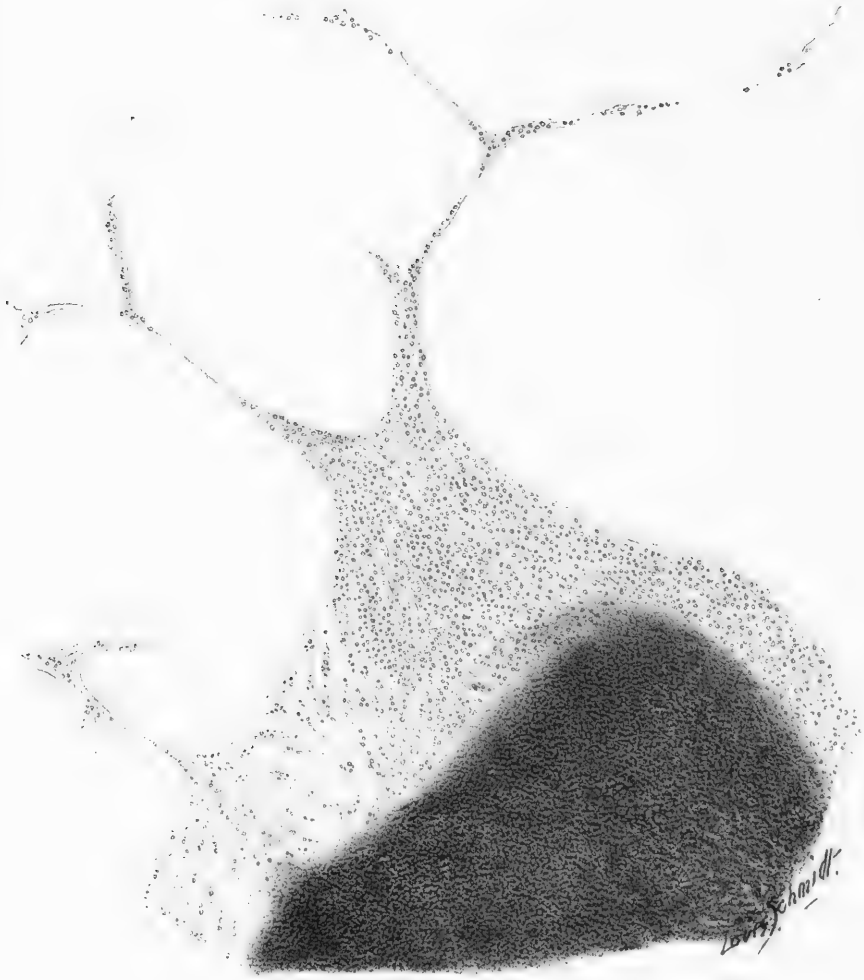

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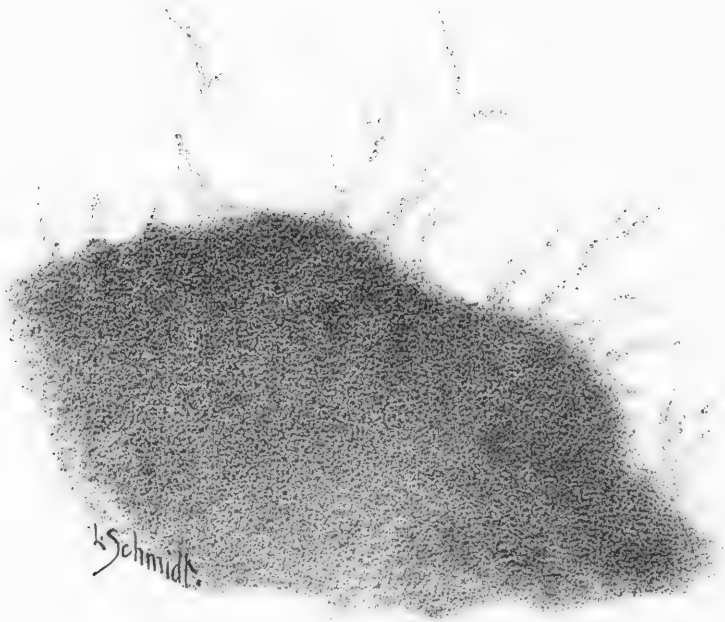

PLATE 48.

Five day cultures in plasma of thyroid gland, from an adult cat.
FIG. 15. The development of elongated connective tissue cells.
FIG. 16. Epithelial cells (another culture). No connective tissue cells.

PLATE 49.

A three day culture of liver from a sixteen day old chick embryo.
FIG. 17. In serum agar.
FIG. 18. In plasma.

PLATE 50.

A three day culture of corium, in serum agar, from a sixteen day old chick embryo.
FIG. 19. High power magnification.
FIG. 20. Low power magnification.

PLATE 51.

FIG. 21. A five day culture in plasma of adult guinea pig bone marrow. Elongated connective tissue cells.
FIG. 22. A three day culture of liver, in serum plus agar, from a fetal cat.

PLATE 52.

FIG. 23. Connective tissue cells spreading along the cover-glass in a culture in serum of a sixteen day chick heart.
FIG. 24. A five day culture in plasma of hypophysis of an adult cat.

PLATE 53.

FIG. 25. A three day culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was heated previously for one half hour at 58° C.

PLATE 54.

FIG. 26. A three day old culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was unheated.

THE RELATIVE PREVALENCE OF HUMAN AND BOVINE TYPES OF TUBERCLE BACILLI IN BONE AND JOINT TUBERCULOSIS OCCURRING IN CHILDREN.*

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INTRODUCTION.

An enormous amount of work has been done to discover the part which the bovine bacillus plays in human tuberculosis, and I wish to record some interesting facts which were revealed in this connection by a study of a consecutive series of cases of bone and joint tuberculosis.

With three exceptions the cases were limited to children twelve years of age and under. Geographically speaking, the subjects inhabited a comparatively localized area,—Edinburgh and its neighborhood; a large number came from the Royal Hospital for Sick Children. Without exception the material was obtained by operation. Out of a total of seventy, thirty-nine were cases of joint disease, thirty-one of bone disease. The series has special characters which distinguish those on which work of a similar kind previously recorded has been based; the numbers are sufficiently great to allow definite conclusions to be drawn, and the age limit is one of the most important features.

It is now recognized that by means of certain tests it is possible to distinguish definitely between the bacilli of human and bovine tuberculosis. By work done along such distinguishing lines, the organismal cause of every case was systematically investigated and ultimately relegated to its special class. I shall now record the result of this investigation and a criticism of its bearing.

* Received for publication, June 6, 1912.

TECHNIQUE.

Isolation of the Bacillus.—No attempt was made to isolate the bacillus by direct cultivation. Guinea pigs were inoculated with the diseased material; tuberculous bone or synovial membrane was implanted *en masse* beneath the skin of the flank. An animal so infected was permitted to live for four or six weeks; during this time the condition of the animal was noted and a careful weight history kept. At the end of the period the animal was killed and from the tuberculous organs, more especially the glands and the spleen, cultures were made upon suitable media. The most successful cultures were made from the caseous interiors of diseased glands, but any tuberculous tissue when thoroughly broken down is suitable.

Cultivation of the Bacillus.—Many varieties of culture media were experimented with, but by experience it was found that two were most reliable, plain egg medium (Dorset (1)) and glycerin egg medium (Lubenau (2)). To these two standard media was added a third, blood egg medium, for which the surface of the plain egg medium was smeared with a small quantity of sterile human blood. The bacillus grows more readily if this last detail is observed.

The diseased material was rubbed upon the surface of the culture tubes with some sterile flat instrument; a small platinum spud is one of the most suitable. Tubes so inoculated were sealed with paraffin to prevent evaporation and to diminish the possibility of infection by moulds. About ten days after inoculation a growth was usually apparent.

Differentiation of the Bacilli.—With the successful growth of the organism in pure culture, the first stage of the investigation might be said to have been completed, and it remained to form an opinion as to which class the organism belonged. To do so the organism was submitted to a series of tests.

A number of tests were introduced; some of them were suggestive in their significance, while others were in my view absolute. As a routine practice I have employed five: (*a*) the original culture test; (*b*) the morphological test; (*c*) the special culture test; (*d*) Theobald Smith's test; (*e*) the inoculation test.

(*a*) *Original Culture Test.*—Smith (3) noted cultural distinctions between different strains of tubercle bacilli when they were grown upon blood serum. Ravenal (4) described similar distinctions upon glycerin agar, and Dorset upon egg medium. The British Royal Commission (5) found such a constant distinction that they formulated two classes; a eugonic, or readily growing class, and a dysgonic, or slowly growing class. To the first the great majority of human bacilli belong. Bovine bacilli are most suitably described under the second heading. This test cannot be considered absolute because a margin of error exists in those cases which are on the border line between eugony and dysogony, but it is useful in so far as it forms the first clue. A rapidly growing and profuse culture is likely to be human in type, while a weak and scanty growth is in all probability bovine.

The fallacies of the test have been discussed by Rabinowitsch (6), Fibiger and Jensen (7), Park and Krumwiede (8).

(*b*) *Morphological Test.*—At one time it was believed that structural differences between the strains of tubercle bacilli might prove of value as a distinguishing test. The human bacillus is usually considered to be slender, regular in shape, and long, while the bovine bacillus is squat and thick (Kossel, Weber, and Heuss (9)). If these differences were constant the test might be a valuable one, but if the test is to be of any assistance the bacilli must be observed in an early and original culture; prolonged residence or multiple subculture tends to produce a common morphological mean. Dorset affirms that he found no such distinctive features between the bacilli, and his views are in agreement with those of Wolbach and Ernst (10).

If the observer is careful to examine the early and original culture, the test is useful as a suggestive one.

In tubercle bacilli there has been frequently noted the presence of deeply staining, spore-like bodies, and they have been described in considerable detail by Coppen-Jones (11). I have found these occurring most constantly in bacilli from a human source, and their presence is best demonstrated by the use of Much's stain.

(*c*) *Special Culture Test.*—The presence of glycerin has a restraining effect upon the growth of the bovine bacillus (Moeller

(12)), but it has rather a stimulating effect upon the growth of the human bacillus (Cobbett (13)). The glycerin is best incorporated in such a medium as glycerin egg. This test must be employed from primary cultures, as the vegetative capacities of the bovine bacillus increase upon frequent subculturing. My experience has been that it constitutes a most valuable test. One may say that all cultures growing luxuriantly from the beginning upon glycerin egg medium are of the human type, while cultures growing sparsely or even not at all on this medium are bovine in character.

(*d*) *Theobald Smith's Test.*—By inoculating glycerin bouillon medium it is possible to produce upon the surface a pellicular growth of tubercle. The rate of growth of the pellicle is an indication of the type of bacillus, but the development of the pellicle gives rise to a change in reaction, which is valuable as a distinguishing test. Before the medium is inoculated its acidity is carefully estimated by titration and made up to a standard reaction by adding 0.05 N/5 of hydrochloric acid to glycerin bouillon neutral to litmus. As the organism grows and the pellicle develops, in the human case the acidity increases, while in the instance of the bovine bacillus, the acidity for a time diminishes and the medium may even become alkaline. It is recognized that the reaction actually depends upon the different rates of growth of the two varieties of bacilli.

Theobald Smith (14) introduced the test, and in a number of cases he found it thoroughly reliable. Bang (15) tested the reaction upon twelve strains of the avian bacillus, using four human and bovine bacilli as controls; he found the reaction constant and he found moreover that the avian bacillus had the same reaction curve as the bovine bacillus.

I applied the test in eleven cases and my results, so far as they go, support Smith's view as to the value of the test. The main difficulty of the reaction lies in obtaining a suitable surface pellicle. The best way to obtain it is to grow the organism primarily upon a tube of egg medium, with a small quantity of glycerin bouillon in the bottom of the tube. As the organism grows it extends from the solid medium over the surface of the bouillon in the form of a pellicle; this pellicle may later be transferred to the Ehrlenmeyer

flask in which the test medium is stored (Smith). If it is impossible to obtain such a pellicle, it is possible to form one upon the surface of the medium; this is done by loading the loop of a platinum needle with the dried culture and gradually scattering it over the surface. It does not sink, but remains as a thin pellicle.

From a medium so inoculated five cubic centimeters of fluid are removed every ten days, and the reaction estimated by titration. Smith recommends that the medium be titrated when hot; before titration it ought to be diluted to 10 per cent. of its usual strength. The titration results may be graphically demonstrated by plotting them upon a curve.

(*e*) *Inoculation Test.*—It has long been noted that tuberculous material from different sources varied in its effects upon bovines and upon man. Villemin (16) observed the fact, and Smith may be said to have formulated it into a test; he published articles on the subject in 1896 (3) and 1898 (17).

The test may be stated as follows: If a rabbit with an average weight of 2,000 grams is inoculated with a small known quantity of tubercle bacilli it will react to the inoculation in various ways. If a human bacillus is employed, the resulting lesions are small and few in number, and after a time they show a tendency to undergo retrogression. If death occurs, it is usually more than six months after the original inoculation, and frequently tuberculosis is not the cause of death. If a bovine bacillus is used, an acute disseminated tuberculosis develops which is rapidly fatal.

I began my investigation as to the exact amount of tubercle which ought to be introduced by performing a series of ten experiments, and I injected one milligram of tubercle intravenously. A second series of thirty experiments was done, and 0.1 of a milligram was employed. Different strains were employed in each series. Finally, the foregoing forty experiments were repeated, and 0.01 of a milligram was used. In these three series the results corresponded, but I have no hesitation in recommending 0.01 of a milligram as the most satisfactory amount for intravenous inoculation. This does not mean that the use of larger amounts would introduce any ambiguity of result, but the employment of the smallest dose necessarily presupposes a corresponding degree of delicacy in the test. The

TABLE I.

Details of case.						Characters of organism.					
No.	Case.	Age in yrs.	Disease.	Family history.	Infantile milk supply.	Culture test.	Morphological test.	Glycerin test.	Smith's test.	Inoculation test.	General conclusion.
1	H. M.	$10\frac{11}{12}$	T. B. Hip	T. B. Pulmonary	Breast fed	Human	Human	Human	Human	Human	Human
2	A. J.	$2\frac{1}{2}$	T. B. Hip	T. B. Pulmonary	Breast fed	Human	?	Human	Human	Human	Human
3	M. C.	6	T. B. Hip	T. B. Pulmonary	Breast fed	Human	Human	Human	Human	Human	Human
4	W. B.	$7\frac{1}{2}$	T. B. Ulna	——[1]	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
5	J. R.	7	T. B. Knee	——	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
6	B. P.	$3\frac{1}{2}$	T. B. Elbow	——	Breast fed	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
7	M. L.	$7\frac{3}{12}$	T. B. Knee	——	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
8	M. C.	10	T. B. Dactylitis	T. B. Pulmonary	Breast fed	Human	?	Human	Human	Human	Human
9	J. F.	$\frac{11}{12}$	T. B. Tibia	——	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
10	D. D.	5	T. B. Knee	T. B. Pulmonary	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
11	J. G.	$1\frac{7}{12}$	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	Bovine	Bovine	Bovine
12	J. Y.	3	T. B. Ankle	T. B. Pulmonary	Cow's milk	Human	?	Human	x[2]	Human	Human
13	A. M.	$2\frac{10}{12}$	T. B. Femur	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
14	H. J.	$6\frac{5}{12}$	T. B. Tarsus	——	Breast fed	Human	Human	Human	x	Human	Human
15	G. Y.	$2\frac{10}{12}$	T. B. Hip	——	Cow's milk	Bovine	Bovine	?	x	Bovine	Bovine
16	M. G.	$\frac{9}{12}$	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
17	M. S.	$\frac{10}{12}$	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
18	J. B.	3	T. B. Knee	——	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
19	A. K.	$1\frac{7}{12}$	T. B. Dactylitis	——	Cow's milk[3]	Bovine	Bovine	Bovine	x	Bovine	Bovine
20	G. S.	$4\frac{7}{12}$	T. B. Tibia	T. B. Pulmonary	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
21	K. R.	3	T. B. Skull	——	Breast fed	Human	Human	Human	x	Human	Human
22	W. B.	$2\frac{1}{2}$	T. B. Dactylitis	——	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
23	D. D.	$1\frac{7}{12}$	T. B. Ankle	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
24	A. N.	$1\frac{6}{12}$	T. B. Humerus	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
25	W. B.	8	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
26	J. P.	6	T. B. Knee	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
27	J. R.	$2\frac{1}{3}$	T. B. Ankle	T. B. Lesions	Breast fed	Bovine	?	Bovine	x	Bovine	Bovine
28	G. N.	3	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
29	A. M.	2	T. B. Ulna	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
30	M. P.	4	T. B. Dactylitis	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
31	G. B.	$10\frac{11}{12}$	T. B. Elbow	——	Cow's milk	Human	Human	Human	x	Human	Human
32	M. E.	$1\frac{5}{12}$	T. B. Tarsus	——	Cow's milk	Human	Human	Human	x	Bovine	Human and bovine[4]
33	D. A.	$1\frac{3}{12}$	T. B. Knee	T. B. Pulmonary	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
34	A. D.	7	T. B. Ilium	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
35	M. D.	3	T. B. Tarsus	——	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
36	D. D.	$1\frac{5}{12}$	T. B. Ankle	T. B. Pulmonary	Breast fed	?	?	Human	x	Human	Human
37	J. C.	10	T. B. Malar	T. B. Pulmonary	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine

38	J. R.	1 3/12	T. B. Tibia	———	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
39	J. C.	8	T. B. Hip	T. B. Gland	Breast fed	Human	?	Human	x	Human	Human
40	A. M.	2 8/12	T. B. Elbow	———	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
41	W. C.	3	T. B. Femur	T. B. Pulmonary	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
42	W. B.	7 1/12	T. B. Joint	T. B. Pulmonary	Cow's milk	Human	Human	Human	x	Human	Human
43	J. F.	1 5/12	T. B. Hip	———	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
44	D. S.	4	T. B. Hip	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
45	W. W.	2 11/12	T. B. Skull	———	Cow's milk	Human	Human	Human	x	Bovine	Human and bovine[4]
46	W. S.	2	T. B. Hip	———	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
47	H. M.	10 1/12	T. B. Hip	T. B. Pulmonary	Breast fed	Bovine	?	Bovine	x	Bovine	Bovine
48	H. C.	7	T. B. Hip	———	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
49	P. M.	1 3/12	T. B. Hip	T. B. Pulmonary	Cow's milk	Human	Mixed	Human	x	Bovine	Bovine and human[4]
50	J. M.	5	T. B. Knee	T. B. Meningitis	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
51	A. D.	4	T. B. Femur	———	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
52	J. F.	5 8/12	T. B. Hip	———	Breast fed	Human	Human	Human	x	Human	Human
53	P. M.	3 1/2	T. B. Dactylitis	———	Breast fed	Human	Human	Human	x	Human	Human
54	R. S.	3 1/2	T. B. Dactylitis	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
55	K. S.	7	T. B. Knee	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
56	J. F.	3	T. B. Hip	———	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
57	R. E.	5	T. B. Ankle	T. B. Pulmonary	Breast fed	?	Human	Human	x	Human	Human
58	B. S. C.	5	T. B. Femur	———	Breast fed	Human	Human	Human	x	Human	Human
59	J. R.	4	T. B. Hip	———	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine

												bovine[4]
33	D. A.	$1\frac{8}{12}$	T. B. Knee	T. B. Pulmonary	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
34	A. D.	7	T. B. Ilium	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
35	M. D.	3	T. B. Tarsus	——	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
36	D. D.	$1\frac{5}{12}$	T. B. Ankle	T. B. Pulmonary	Breast fed	?	?	Human	x	Human	Human
37	J. G.	12	T. B. Malar	T. B. Pulmonary	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
38	J. R.	$1\frac{4}{12}$	T. B. Tibia	——	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
39	J. C.	8	T. B. Hip	T. B. Gland	Breast fed	Human	?	Human	x	Human	Human
40	A. M.	$2\frac{8}{12}$	T. B. Elbow	——	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
41	W. C.	3	T. B. Femur	T. B. Pulmonary	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
42	W. B.	$7\frac{1}{12}$	T. B. Joint	T. B. Pulmonary	Cow's milk	Human	Human	Human	x	Human	Human
43	J. F.	$1\frac{8}{12}$	T. B. Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
44	D. S.	4	T. B. Hip	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
45	W. W.	$2\frac{11}{12}$	T. B. Skull	——	Cow's milk	Human	Human	Human	x	Bovine	Human and bovine[4]
46	W. S.	2	T. B. Hip	——	Cow's milk	Bovine	?	Bovine	x	Bovine	Bovine
47	H. M.	$10\frac{11}{12}$	T. B. Hip	T. B. Pulmonary	Breast fed	Bovine	?	Bovine	x	Bovine	Bovine
48	H. C.	7	T. B. Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
49	P. M.	$1\frac{3}{12}$	T. B. Hip	T. B. Pulmonary	Cow's milk	Human	Mixed	Human	x	Bovine	Bovine and human[4]
50	J. M.	5	T. B. Knee	T. B. Meningitis	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
51	A. D.	4	T. B. Femur	——	Breast fed	Bovine	Bovine	Bovine	x	Bovine	Bovine
52	J. F.	$5\frac{8}{12}$	T. B. Hip	——	Breast fed	Human	Human	Human	x	Human	Human
53	P. M.	$3\frac{1}{2}$	T. B. Dactylitis	——	Breast fed	Human	Human	Human	x	Human	Human
54	R. S.	$3\frac{1}{2}$	T. B. Dactylitis	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
55	K. S.	7	T. B. Knee	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
56	J. F.	3	T. B. Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
57	R. E.	5	T. B. Ankle	T. B. Pulmonary	Breast fed	?	Human	Human	x	Human	Human
58	B. S. C.	5	T. B. Femur	——	Breast fed	Human	Human	Human	x	Human	Human
59	J. R.	4	T. B. Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
60	F. F.	30	T B. Dactylitis	T. B.	?	Human	Human	Human	x	Human	Human
61	N. C.	25	T. B. Vertebral	——	?	Human	Human	Human	x	Human	Human
62	A. S	24	T. B. Ankle	T B. Pulmonary	?	Human	Human	Human	x	Human	Human
63	P. D.	3	T. B. Femur	T. B. Gland	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
64	J R.	4	T. B. Tibia	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
65	R. C.	$8\frac{1}{2}$	T. B. Ankle	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human
66	S. H.	4	T. B. Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
67	D. S.	2	T. B. Os. Culture	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
68	W. B.	8	T. B Hip	——	Cow's milk	Bovine	Bovine	Bovine	x	Bovine	Bovine
69	J. F.	4	T. B. Hip	——	Breast fed	Human	Human	Human	x	Human	Human
70	R. E.	5	T. B. Ankle	T. B. Pulmonary	Breast fed	Human	Human	Human	x	Human	Human

[1] (—) denotes the absence of evidence of tuberculosis in the family history.

[2] (x) denotes that the test was not applied.

[3] Tubercle bacilli were actually found in the cow's milk by Dr. Mitchell, working in this laboratory.

[4] The conclusion that two varieties of organism occurred in this case was based upon the reaction of the glycerin test (human) and the inoculation test (bovine).

amount for inoculation may be obtained by the volumetric method or by weight. The volumetric method is that recommended by the British Commission (18):

"The doses of culture have been estimated volumetrically in the following way: the growth from the surface of each serum tube is scraped off with a platinum spatula and emulsified with a drop or two of sterile salt solution between sterilized ground glass plates; the thick resulting emulsion is diluted with salt solution and run into a sterile test tube. . . . The emulsion is vigorously shaken and put aside for half an hour to allow all the large clumps of bacilli to settle. The supernatant fluid is then decanted into a separate test tube and accurately graduated capillary tubes are immediately filled from it. The tubes after being sealed at both ends are centrifuged until all the culture has accumulated at the bottom of each tube in the form of a small column; the length of the column of bacilli and that of the combined columns of bacilli and fluid in each tube are then carefully measured, the average is taken and expressed as a ratio. Having determined the relative amounts of bacilli and fluid with emulsion, the amount of emulsion necessary for a given dose can be readily calculated. For example, if a dose of 10 mgm. is required and the emulsion contains 1 volume of bacilli in 40, 0.4 cubic centimeter is taken."

In the weight estimation, the bacilli are scraped off the culture, and as a preliminary are dried over sulphuric acid; the bacterial mass is weighed upon a delicate balance to the extent of one milligram. This amount of culture is emulsified, and the emulsion diluted to the extent of one hundred cubic centimeters with sterile saline. One cubic centimeter of this amount is inoculated intravenously into the ear vein. In dealing with such small amounts a considerable margin of error must necessarily be allowed for; with the use of 0.01 of a milligram of bacilli the test is absolute.

ANALYSIS OF CASES.

Each case was systematically worked out upon the lines which I have detailed, and evidence of the identity of the bacillus accumulated until a final conclusion could be reached regarding the class to which the organism belonged. It would be tedious and unnecessary to state the progress of separation in each individual case, and I have therefore tabulated the facts, evidence, and result in the following tables.

There were seventy cases in all; in individual columns are given the necessary facts regarding disease, age, family history, and milk supply. The result of each test is given and the final conclusion stated (table I).

DISCUSSION OF RESULTS.

Type of Organism.—Bacteriological studies of bone and joint tuberculosis have been recorded by the British Royal Commission (19), fourteen cases; Park and Krumwiede (8), seventeen cases; and Buckhardt (20), twenty-nine cases.

Of the cases investigated by the Commission one was that of a girl of fourteen years; the others were adults. The Commission results showed that thirteen cases were due to the human bacillus; the last of the series contained both the human and the bovine bacillus.

Park and Krumwiede's cases were without exception due to the human bacillus. Six of the cases were children under five years of age; ten were children whose ages ranged from five to sixteen years; a single case was an adult.

In Buckhardt's series, thirteen cases were under twelve years of age; ten ranged from thirteen to twenty-one years; the remainder were adults. Buckhardt found that three cases of joint tuberculosis contained the bovine bacillus; the respective ages were eleven, nineteen, and fourteen years.

Kossel, Weber, and Heuss examined a series of thirty-six cases; twelve cases were obtained from adults and each contained the human bacillus. Twelve cases were children from five to sixteen years old; one of these was due to the bovine bacillus. Thirteen cases of children under five years were due to the human bacillus.

A small series of investigations (three cases) has been carried out by Henschen, Jundell, and Svensson (21). All the cases examined were human in character.

The results which I have to offer are in many respects different from those which I have quoted. Out of a total of seventy investigations, in forty-one instances the bovine bacillus was present, in twenty-six cases the human bacillus, and from the remaining three cases both the human and bovine bacilli were isolated. The outstanding feature is the large proportion of cases which owed their origin to infection by the bovine bacillus. Correlated with these results and to a certain extent explanatory of them, are questions of age, family history, milk supply, and environment; and I believe that these are the special factors which explain the differences presented by my series as compared with the results of other investigators.

Age of Patients.—With three exceptions the cases were those of children twelve years of age and under. In no experimental work which I have seen has the age limit been a feature. To my

mind it is valuable to have a series confined to the early years of life, for two reasons. The first is that a large proportion of children of this age are nourished in whole or in part upon cow's milk. The second is that the capacity for resistance on the part of the mesenteric lymphatic system is less in early childhood than in later life. That this is recognized by clinicians is borne out by numerous investigators. Dr. John Thomson (22) has pointed out that the percentage of abdominal tuberculosis among children admitted to the Edinburgh Sick Children's Hospital, from which many of my cases were derived, ranges from 3 to 4 per cent., while in Glasgow the percentage is even higher; namely, 4 to 5 per cent. The correlation between these observations and my own points to the prevalence of an infection of the milk by the bacillus of bovine tuberculosis.

I have tabulated the cases according to age and the type of infection (table II). It will be noted that during the first three years of life the majority of the cases occurring were due to the bacillus of bovine tuberculosis.

TABLE II.

Age.	No. of cases.	Bovine bacillus.	Human bacillus.	Bovine and human bacilli.
To 1 year	4	4	—	—
Years 1–2	12	8	2	2
Years 2–3	15	11	3	1
Years 3–4	10	6	4	—
Years 4–5	6	3	3	—
Years 5–6	3	—	3	—
Years 6–7	5	3	2	—
Years 7–8	6	4	2	—
Years 8–9	1	—	1	—
Years 9–10	1	—	1	—
Years 10–11	3	1	2	—
Years 11–12	1	1	—	—
Adults	3	—	3	—

It is interesting to note that the cases of adult bone tuberculosis were due to a human infection.

Family History.—In every case investigated, the family history was carefully inquired into and it was found that in twenty-one instances there was a history of pulmonary tuberculosis having occurred in some member of the patient's family; and in 50 per cent. of these cases the child was actually living in contact with a consumptive when the bone lesion developed. Increased importance

is given to the fact when it is found that fifteen of these cases, or 71 per cent., were due to the bacillus of human tuberculosis. I have no doubt that in such cases there was a direct infection from the consumptive to the child. This is a fact which might be of considerable use as an argument for the compulsory isolation of phthisis cases.

I have tabulated these facts in table III.

TABLE III.

	Human bacillus.	Bovine bacillus.	Percentage of human strains.
History of tuberculosis in family.....	15	6	71 per cent.
No history of tuberculosis in family.....	9	43	17 per cent.

Milk Supply.—With such a considerable proportion of cases due to infection by the bovine bacillus, the question of milk supply becomes of great importance. There can be no doubt that milk was the medium through which infection occurred. In the list which I have compiled there are a number of children less than one year old; in each of these cases the bovine bacillus was the organism present and each had been entirely nourished upon cow's milk. The same lesson is obvious throughout; in the group of cases, the ages of which range from one to two years, twelve in number, there are eight which owed their origin to the bovine bacillus, and every one of the number had been nourished from birth upon cow's milk. Two cases in this series contained both human and bovine bacilli; each had been nourished upon cow's milk and each showed a family history of pulmonary tuberculosis. It was instructive to note that in no case investigated had any attempt been made to ensure the purity or sterility of the milk.

CONCLUSIONS.

The all important point revealed by the investigation is the fact that a large proportion of bone and joint tuberculosis occurring in children in Edinburgh owes its origin to infection by the bovine bacillus.

The bovine bacillus is introduced into the system practically by

one route only, that of ingestion, and the medium with which it is ingested is cow's milk. It is not my intention to criticize in any way the existing conditions of milk supply. I have furnished proof of what is actually occurring and no one will deny that the evil is a remediable one.

In those cases in which the human bacillus was present, a considerable proportion showed a definite history of pulmonary tuberculosis affecting a co-resident, and every fact went to prove that the infection had been a direct one from patient to child.

A complete distinction can be drawn between human and bovine bacilli, and the distinction is best secured by subjecting the organism to a series of tests such as I have detailed.

The subject is one which ought to be investigated in a series of different localities. It is possible that the *locus* may be a factor in the explanation of the difference between the above results and those of other observers.

In conclusion I wish to acknowledge my indebtedness to Mr. H. J. Stiles, Surgeon to the Sick Children's Hospital, for permission to obtain and use material, and for his valuable assistance and advice; to Dr. James Ritchie, Superintendent of the Laboratory of the Royal College of Physicians, Edinburgh, for much help and guidance; to the Trustees of the McCunn Research Fund and to the Carnegie Trustees for the funds which defrayed the expenses of the investigation.

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ACUTE VASCULAR LESIONS IN MICE FOLLOWING INJECTIONS OF PNEUMOCOCCI.*

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Plates 55–58.

In a previous report by one of us (1) on the comparative virulence of pneumococci at different stages of pneumonia, it was noted that mice dying after an injection of living pneumococci showed hemorrhage in the pleural or pericardial cavities, consisting of fresh blood clots varying in size from a wheat grain to a large pea. Among eighty-four mice that succumbed, twenty-four showed such a blood clot. The duration of life after inoculation varied from one to twenty days. The cause of the hemorrhage was traced by serial sections of one case to the rupture of a large branch of the pulmonary artery, besides which a markedly degenerated area in the transverse arch of the aorta and the base of one of the large branches arising from it (case I of this report, figures 1 and 2) was found.

The question then arose as to the constancy of these findings and whether the arterial changes were caused by the pneumococci injected or whether the vessels were ruptured by some trauma during life or some accident at autopsy, or possibly whether they were the result of some preëxisting disease among the mice.

Twenty-one mice were studied with reference to these points. Fourteen had been injected with living pneumococci, of which ten showed the hemorrhagic condition at autopsy; four showed no hemorrhage but were examined for lesions of the vessels; three died spontaneously, never having been inoculated, and one mouse was presumably healthy, having been killed by a blow on the head.

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Finally, three succumbed to inoculation of dead pneumococci, among which one showed hemorrhage.

We have examined one hundred and eighteen mice inoculated with pneumococci during the past year with reference to the hemorrhage described. Of these, eleven, or 9.3 per cent., showed the large hemorrhages, while among our previous series 28.5 per cent. had shown the condition. The discrepancy is probably to be explained by the difference in dosage. In the recent work small doses of highly virulent cultures have been given, so that a large proportion of these animals developed septicemia and died acutely. With larger doses of less virulent strains the animals often survived for many days. It is among these examples of delayed deaths occurring suddenly after a period in which the mice appear well that the hemorrhages are usually found.

The bacteriological methods have been described in the paper referred to (1) and need not be repeated here. The autopsies were conducted with great care, and in cases of intrathoracic hemorrhages special precautions were observed to prevent any injury or disturbance of the relations of the thoracic viscera. The ventral wall of the thorax was removed and the whole body immersed in fixative which was in some cases Zenker's fluid, in others 10 per cent. formalin. After the tissues were thoroughly fixed and hardened, the viscera were removed *en masse* and embedded in celloidin. In about half the cases only the organs above the diaphragm were so treated; in the others the upper abdominal organs were also included. Sections were cut twelve to eighteen microns thick and every tenth or fifteenth section was stained and mounted. In some cases every sixth section from the important part of the block was studied. The series were usually stained in hematoxylin and eosin and extra sections were colored by Weigert's method for elastic tissue. In a few instances the series was first stained in orcein by Unna's method and subsequently in hematoxylin and eosin. In one case frozen sections were cut from formalin-fixed tissue and stained for fat by Herxheimer's scarlet R method.

HISTOLOGY.

The findings in each case are summarized in the accompanying table (table I). The general features may be described as follows:

1. Ten mice injected with living pneumococci showed the hemorrhages. Seven of these showed on section rupture of the ascending aorta. In two of these seven the pulmonary artery was ruptured also. In the eighth mouse the hemorrhage arose from rupture of the pulmonary artery alone and in the ninth the source of hemorrhage was not precisely discovered, but the position of the blood clot indicated an origin from the pulmonary artery. The source of the hemorrhage in the tenth mouse was not determined.

2. Four mice injected with living pneumococci succumbed, but without showing the hemorrhage. One of these showed in section rupture of the transverse arch of the aorta with hemorrhage into the mediastinal tissues. Two showed small hemorrhage into the pleural cavity and lungs, and the other one showed no hemorrhage at all.

3. The three mice succumbing spontaneously showed no hemorrhage.

4. Three mice succumbed to injections of dead pneumococci. Two showed no hemorrhage, while one mouse exhibited rupture of the ascending aorta attended with hemorrhage.

The lesion appears usually merely as a break with rather ragged edges in one side of the vessel, about which a platelet thrombus has formed, and the whole is embedded in a dense mass of extravasated blood (figures 3 and 4). In extreme cases there is complete disappearance of the aorta through many sections, the site of the vessel being indicated by a ring of agglutinated platelets.

Above and below the area of rupture the vessel may show no microscopical change. The lumen and thickness of wall remain unaltered and the muscle cells and elastic fibers stain well. The space left by the disappearance of the vessel wall is usually larger in the center, becoming gradually smaller toward each extremity until the broken ends unite. At this point it is found that the elastic fibers remain visible and stainable, although perhaps ruptured and stretched (figure 5), forming an irregular bridge across the breach, while the muscle cells have entirely disappeared. In the most ex-

TABLE I.

Case no.	Material injected.	Duration of life.	Autopsy.	Vascular lesions.	Other lesions.
I	20,000,000 pneumococci subcutaneously.	10 days	Hemorrhage, pleural cavity.	Ruptured pulmonary artery; degenerated and ruptured transverse arch and carotid; degenerated vein.	
II	Mouse killed.		No hemorrhage.	Slight hemorrhages in pericardium and pleura; vessels and heart normal.	Peribronchial lymphoid infiltration.
III	20,000,000 pneumococci intraperitoneally.	5 days	Hemorrhage, right pleura; cultures −.	Ruptured pulmonary artery (small).	Hemorrhages in lungs.
IV	80,000,000 pneumococci intraperitoneally.	5 days	No hemorrhage; cultures −.	Small hemorrhages around pulmonary vessels; no definite vascular lesion.	Hemorrhages in lungs.
V	20,000,000 pneumococci intraperitoneally.	2 days	No hemorrhage; peritonitis; cultures +.	No vascular lesion; no hemorrhage.	Pulmonary congestion.
VI	?	6 days	No hemorrhage; congestion-lung.	Erosion of pulmonary vein by bronchopneumonia.	Pneumonia; pleuritis; mediastinitis.
VII	80,000,000 pneumococci intraperitoneally.	6 days	No hemorrhage; smears −; cultures +.	Rupture of transverse aortic arch (small).	Hemorrhages in lungs; pericarditis; pleuritis; mediastinitis.
VIII	200,000 pneumococci subcutaneously.	5 days	Hemorrhage, pericardium.	Rupture of ascending aorta.	Peribronchial lymphoid infiltration.
IX	Spontaneous death.		No hemorrhage.	Slight hyaline change in aorta; myocarditis.	Liver parasites.
X	Spontaneous death.		No hemorrhage.	No lesion; no hemorrhage.	Peribronchial lymphoid infiltration; peritonitis.
XI	2 injections of 100,000 and 20,000,000 pneumococci.	10 days	Large hemorrhage, pericardium; cultures −.	Rupture of ascending aorta (extreme).	Focal necroses in liver.
XII	24,000 pneumococci subcutaneously.	5 days	Small hemorrhage, pericardium.	Rupture of ascending aorta; dissecting aneurysms; rupture of jugular vein.	Peribronchial lymphoid infiltration; focal necroses in liver.
XIII	100,000 pneumococci subcutaneously.	5 days	Small hemorrhage, pericardium.	Ruptures of ascending aorta and pulmonary artery.	
XIV	5,000 pneumococci.	5 days	Small hemorrhage, pleura.	No lesion (partial examination, frozen sections).	

Case no.	Material injected	Duration of life.	Autopsy.	Vascular lesions.	Other lesions.
XV	30,000,000 pneumococci subcutaneously.	4 days	Very large hemorrhage.	Rupture of ascending aorta (extreme); dissecting aneurysms; pericarditis, endocarditis; myocarditis.	Pleuritis; peritonitis; focal necroses in liver.
XVI	Dead pneumococci.	2 days	Small hemorrhage, pericardium.	Rupture of ascending aorta; degenerated vein.	Peribronchial lymphoid infiltration;
XVII	2 injections of dead pneumococci.	7 days	No hemorrhage.	No lesion.	Widespread acute inflammation.
XVIII	2 injectious of dead pneumococci.	7 days	No hemorrhage.	No lesion. Pericarditis.	Peribronchial lymphoid infiltration focal necroses in liver.
XIX	Spontaneous death.		No hemorrhage.	No lesion.	Tuberculosis (?) of liver.
XX	80,000,0000 pneumococci subcutaneously.	5 days	Large hemorrhage.	Rupture of ascending aorta; dissecting aneurysm; myocarditis.	Peribronchial lymphoid infiltration; focal necroses in liver; parasite.
XXI	320,000,000 pneumococci intraperitoneally.	2 days	Hemorrhage, pericardium and pleura.	Rupture of pulmonary artery (?).	

treme part of the lesion, remnants of elastic fibers may sometimes be demonstrated by special stains, although no sign of the arterial wall can be brought out with the ordinary stains.

Occasionally, above or below the point of rupture, small dissecting aneurysms are found. In a few cases the aortic wall shows marked degeneration, but preserves its continuity. There is a sharp line of demarcation between the normal tissues and the thinner, almost structureless membrane in which elastic remnants may be found. In case XII this portion was bulged out to form a small sac (figure 6).

The heart muscle appeared normal except for an acute myocarditis that occurred in a number of cases.

The veins are usually entirely normal. In a few instances changes strongly suggestive of degeneration occurred. Sometimes for a short arc of its circumference the vein wall stained poorly and the cell striations and outlines were lost. One such lesion had led to a definite rupture attended with a small hemorrhage.

In and about the vascular lesions there are no indications of an

inflammatory reaction. No organisms could be stained in the sections, and cultures taken from the blood at autopsy are usually sterile. In a number of cases inflammatory exudates in the pleura, pericardium, mediastinum, and lungs existed, but in no case could the pathological condition of the vessels be attributed to an extension of an inflammatory process, save, perhaps, in one instance of the pulmonary vein (case VI).

ETIOLOGY.

We have up to the present found these acute vascular lesions in those mice only which had been inoculated with living or dead pneumococci. None of the mice dying from other causes and examined histologically showed any such lesions. Of the two hundred or more mice used in the medical laboratory this year, not more than a dozen have died spontaneously, so called. The autopsies made on several of these showed no gross hemorrhage. Twelve mice were injected with streptococci in the course of other investigations and no blood clots were found at autopsy.

The evidence would therefore seem, as far as our observations are concerned, to determine the pneumococcus as the cause of the lesions. The negative cultures at autopsy, the absence of bacteria and microscopic inflammatory reaction, and the fact that the same conditions may be produced by injection of the dead microorganisms suggest that the pathological condition produced is not an arteritis but a toxic degeneration of the vessel wall produced by the poisons of the injected pneumococci.

That bacteria and their products have a definitely harmful effect upon the arteries of certain laboratory animals, for example rabbits, has been shown by Klotz (2), Duval (3), Saltykow (4), and others. Saltykow claims that by the use of bacterial poisons (staphylococci) which are injected repeatedly into rabbits over considerable periods of time, lesions can be produced resembling closely those found present in human arteriosclerosis. Mice, we believe, have not been used before in the study of experimental arterial disease nor have pneumococci received much attention from this point of view. Those who have used mice for the deter-

mination of virulence of pneumococci have probably not been in the habit of making such detailed autopsies as are here carried out.

Although the findings in mice bear some resemblance to the traumatic arterial ruptures and to the spontaneous rupture of the aorta, so called, in man, yet a traumatic origin cannot be assigned for them. The affected vessels are very deeply placed in the mouse, and definite degenerations have been discovered in the thin walls that attend the rupture and extravasation. Besides this, the hemorrhagic condition has been noted in mice inoculated with pneumococci and not in the numerous control animals.

The localization by preference in the ascending aorta may be analogous to the well known predilection of syphilitic aortitis for the same region. We should, perhaps, consider the possibility that the definite, localized degeneration is not of regular occurrence, that the walls of arteries and veins are weakened generally all over the body but without histological change, and that the rupture occurs when the blood pressure exceeds the resistance offered by the weakened vessel and most frequently in the ascending aorta where the pressure is highest. Their occurrence, however, in the pulmonary arteries and in the veins is strongly against this view.

This work may serve to emphasize the importance of the rôle played by infectious diseases and their bacterial poisons in the etiology of arterial and vascular diseases in general. Different writers (5) on arteriosclerosis have called attention from time to time to the factor, but it can scarcely be said to have obtained general acceptance. The poisonous substances are the same, as far as we know, in mouse and man, and the tissues of the arterial walls at least are composed of the same histological elements.

SUMMARY.

Mice dying several days after injections of pneumococci, both living and dead, frequently show at autopsy large intrathoracic hemorrhages.

The histological study of the thoracic organs indicates that there occurs in each case a sharply circumscribed, acute degeneration of the wall of some large vessel, usually the ascending aorta or one of the pulmonary arteries. This degenerated portion is torn out by

the pressure of the blood with almost complete disappearance of the vessel wall, leading to a gross hemorrhage.

A similar change is occasionally found in the walls of the veins which contain cardiac instead of smooth muscle.

We have found this lesion only in mice which had been recently inoculated with pneumococci. Negative cultures at autopsy, the lack of inflammatory reaction, and the occurrence of the conditions after injection of dead pneumococci suggest the cause to be a toxic degeneration of the vessel wall brought about by the poisons of the injected organisms.

We are indebted to Dr. P. W. Clough for several of the animals which died of hemorrhages in the course of his investigations in the medical laboratory.

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EXPLANATION OF PLATES.

Plate 55.

Fig. 1. Case I. Aortic wall at the right; elastic remnants shown in degenerated wall of carotid at the left. Weigert's elastic tissue stain.

Fig. 2. Case I. Pulmonary artery. The light area at the right is a platelet thrombus in which may be seen elastic remnants. Weigert's elastic tissue stain.

FIG. 1.

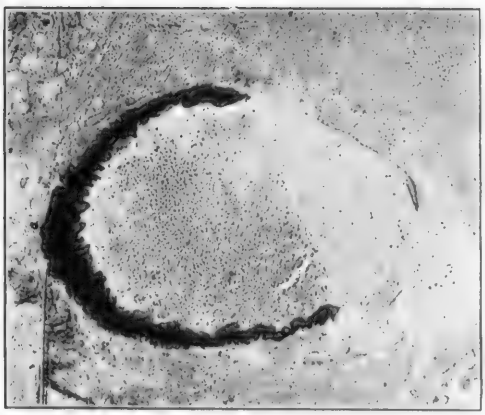

FIG. 2.

FIG. 3.

FIG. 4.

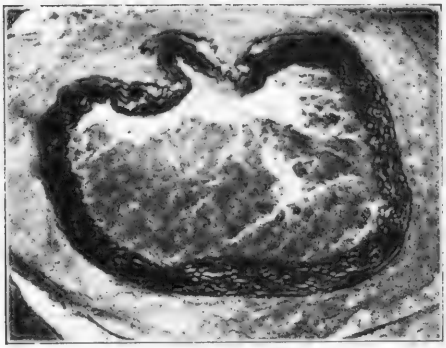

FIG. 5.

FIG. 6.

PLATE 56.

FIG. 3. Case XX. Thoracic organs. Low power; hematoxylin and eosin. H = heart; x = thymus; T = trachea; e = esophagus; $D.A.$ = descending aorta. The ruptured ascending aorta is seen in the center with a light staining platelet thrombus. The dark material throughout the section is extravasated blood.

PLATE 57.

FIG. 4. Case XX. Ascending aorta. Hematoxylin and eosin. Ruptured and stretched elastic fibers bridge the opening in the wall. A thrombus is seen in the lower left corner.

FIG. 5. Case VIII. Ascending aorta. Orcein stain. Below the main lesion.

PLATE 58.

FIG. 6. Case XII. Lower part of the ascending aorta. Hematoxylin and eosin. The degenerated, hyaline portion of the wall is bulged out to form a small aneurysm. Above there is a sharp break in the vessel wall. The blood is held in check by the adventitia.

SINUS STIMULATION AS A FACTOR IN THE RESUSCITATION OF THE HEART.*

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The number of instances now on record of resuscitation of the apparently dead by means of massage of the heart[1] furnishes more than enough justification for the continued practice of this measure, the value of which was first indicated in 1874 by the experiments of Schiff on animals. At the same time the large number of failures to resuscitate by means of this method in cases to all appearances quite as favorable as those revived, demonstrates that there is still much to be accomplished in this direction. Even in the case of animals where every preparation for resuscitation has been made, the heart often cannot be made to beat by means of massage.

An analysis of the causes of cessation of the pumping action of the heart should be the first step toward the clarification of the difficulties in the way of resuscitation. The main possible causes of acute cardiac failure may be described as follows: (1) inhibition; (2) fibrillation of the ventricles; (3) loss of contractility due to (*a*) asphyxia, (*b*) anemia, (*c*) poisons, (*d*) acute dilatation, (*e*) long standing disease of musculature; (4) loss of rhythmicity: (*a*), (*b*), (*c*) as in (3), (*d*) disease of the *primum movens,* together with loss of rhythmicity of subjacent parts; (5) loss of conductivity together with loss of rhythmicity of subjacent parts. It should be stated that the causes mentioned under (3), (4), and (5) are nearly always associated in one and the same case, although it may be not in equal degree.

The possibility of, and the methods available for the resuscita-

* This paper was read before the St. Louis Medical Science Club and published in abstract in its Proceedings. in the *Interstate Medical Journal,* June, 1912. Received for publication, June 19, 1912.

[1] For the literature see Metcalf, C. R., *Boston Med. and Surg. Jour.,* 1909, clxi, 9; and Jurasz, A. T., *München. med. Wchnschr.,* 1911, lviii, 83.

tion of the heart acutely insufficient as the result of one or more of the above mentioned causes may now be considered.[2]

1. Stoppage due to inhibition, such, for example, as occurs in the early stages of chloroform anesthesia, should be best combated by means of cardiac massage. This alone should suffice to maintain the circulation until the inhibition is removed. The administration of atropin might hasten the removal of the inhibition.

2. Fibrillating ventricles, it would seem, are beyond treatment. Garrey[3] has occasionally succeeded in stopping fibrillation of the dog's ventricle by means of stimulation of the vagus nerve. This measure, however, must be resorted to so soon after the inception of fibrillation that it can be of little assistance in the case of man, unless it should be shown that there is a decided difference in this regard between man and the dog. The statement of Arabian[4] to the effect that the high tension alternating current stops fibrillation would seem to require verification.

3, 4, and 5. Failure due to loss of contractility, rhythmicity, and conductivity, when due to general or acute conditions, should be benefited by massage. A majority of the cases of cessation met with clinically is occasioned by this combination. And the fact that a majority of the cases, many of them apparently favorable, cannot be recovered by massage alone, would seem to indicate the need of subsidiary measures. In so far as the heart is concerned, the action of any condition promoting insufficiency through the depression of any one or all of these properties of the heart muscle starts a vicious circle. If, for instance, the prime action be upon contractility, this through the insufficient propulsion of the blood resulting therefrom will in turn depress rhythmicity and conductivity. Even when contractility is supplied by the hand of the operator, rhythmicity or conductivity, or both, may fail to return.

In resuscitation experiments it can easily be shown that massage frequently restores irritability and contractility, but fails to develop spontaneous rhythmicity; the inner stimulus is lacking. In such cases it becomes necessary of course to supply the rhythm or to develop it. Since the contraction of the ventricles alone suffices to maintain an efficient circulation, restoration of the circulation might be accomplished by means of direct stimulation of the ventricles with rhythmical induction shocks.[5] That this procedure is, how-

[2] It is not the purpose of this discussion to describe in detail the methods of resuscitation as applied to the organism as a whole, but merely to recount the general principles for the resuscitation of the heart itself. Therefore no reference is made to the concomitant action of other factors, such, for example, as vasomotor failure.

[3] Garrey, W. E., *Am. Jour. Physiol.*, 1908, xxi, 283.

[4] Arabian, H., *Jahresb. ü. d. Fortschr. d. Physiol.*, 1903, xii, 57.

[5] Special forms of apparatus have been devised for the purpose of supplying such stimuli to the ventricles (Floresco, N., *Jour. de physiol. et de path. gén.*, 1905, vii, 785, 797).

ever, fraught with very great danger every one knows who has worked to any extent with the exposed heart. In the state in which we find them when attempts at resuscitation become necessary, the ventricles are extremely liable to fibrillate, and an induction shock, or a galvanic current of sufficient strength to elicit a contraction, will almost certainly set up fibrillation.[6] It is therefore not safe to supply the rhythm in this way.

The impulses supplied to the ventricles along the normal path,—the auriculo-ventricular bundle,—are not nearly so apt as is direct stimulation to cause the ventricles to fibrillate. In the case of a heart that has stopped beating, it does not, however, always suffice for the supplying of impulses along the usual path merely to start the auricles beating. Owing to lowered conductivity at the auriculo-ventricular junction, which is the part of the heart especially susceptible to unfavorable conditions, impulses originating in a beating auricle may fail to reach the ventricles. Nor is this the only reason for insufficiency of auricular impulses. Owing to the feebleness of impulses from a feebly beating auricle, or to reduced irritability of the ventricular muscle, the auricular impulses may fall below the threshold of the ventricles. In such an event any measure that would make the auricles spontaneously rhythmical, or would increase the strength of their impulses, might prove of very great assistance in the resuscitation of the heart. It is just here that the methods now employed for the resuscitation of the heart could be improved.

In some experiments described in 1910[7] it was found that strips of the auricles of the cat's heart when stimulated tetanically may become spontaneously rhythmical, and that by the same means the rate of an existing beat may be materially increased provided the part stimulated is the sinus region of the heart. Stimulation of other parts of the auricle,—for example, the vault, the right appendix, or the left auricle,—rarely if ever results in the development of

[6] We have had no opportunity to test in this connection the statement of Batelli (*Jahresb. ii. d. Fortschr. d. Physiol.*, 1900, ix, 69) to the effect that an alternating current of 120 volts causes fibrillation, whereas one of 240 volts and 45 cycles does not; indeed that it stops fibrillation and causes the heart to beat normally.

[7] Erlanger, J., *Am. Jour. Physiol.*, 1910–11, xxvii, 87.

a series of beats. Furthermore, it was found that short periods of tetanic stimulation of the sinus region materially increase the carrying power into the less rhythmical regions of the auricle of the impulses originating in the sinus, so that previously quiescent outlying regions may begin to respond to sinus impulses, or a feebly beating outlying region may begin to beat more strongly. It should be added that stimulation of the auricles proper is not without effect. It makes them more responsive to stimuli carried into them from without, the amplitude of the contractions so determined being materially increased thereby. These results raised the hope that attempts at resuscitation of the heart might be materially aided by combining with the procedures now more or less generally used tetanic stimulation of the auricles, particularly of the sinus region.

RESTORATION OF THE NORMAL RHYTHM IN THE PERFUSED HEART.

Observations on the artificially perfused heart have shown clearly the value of this procedure in cases where perfusion, a measure quite comparable in its effects to massage of the heart, fails to develop a normal heart beat. Two illustrative cases will be cited here. In both cases the heart (cat) had fibrillated and the fibrillations had been stopped by temporary perfusion with a 1 per cent. potassium chlorid solution. In one case (experiment 1, series I) upon reperfusion with Locke's solution the ventricles alone began to beat, at a very slow rate. Single induction shocks applied to the sinus region of the auricles with a pair of platinum electrodes caused the heart to respond regularly and in proper sequence to each stimulus, but upon the cessation of stimulation the heart beat resumed its previous state. Then the sinus region was stimulated tetanically. Immediately after the cessation of stimulation, and for some time thereafter the beat of the heart to all appearances was perfectly normal. Soon, however, the sequence underwent a gradual change until the ventricles contracted first, and the auricles almost immediately after them. By means of tetanic stimulation of the sinus the normal sequence was repeatedly restored.

In the other case (experiment 2, series I) after reperfusion with Locke's solution the ventricles alone began to beat. Tetanic stimulation of the sinus region once and for all started the normal se-

quence. Experiments of the same kind have shown that stimulation of other parts of the auricle than the sinus region will not restore the normal sequence of beat.

EXPERIMENTS ON THE EXPOSED HEART IN SITU.

Experiments were then made on the dog's heart, which was exposed in the manner usual for experimental purposes. The effect of the procedure upon the exposed heart was observed, and in addition the pressure in the carotid artery was recorded with a mercury manometer. The stimuli were applied to the sinus region through a pair of platinum electrodes, which in some cases were sewn to the heart, and in others were passed into the auricle through the jugular vein. The animals were completely anesthetized; the preliminary anesthesia in all cases was obtained with morphine and ether. The heart failure was brought on usually by allowing the animal to inhale chloroform through the tracheal cannula until the respirations ceased and by waiting, as a rule, until the heart stopped beating. In some experiments the animals were asphyxiated while under full ether anesthesia by clamping the trachea until the heart had stopped. Then artificial respiration was begun and steps toward resuscitation were taken. These consisted of tetanic stimulation of the sinus and direct massage of the heart applied sometimes at the same time and sometimes separately. Other accessory measures, such as abdominal pressure, the infusion of salt solution, with or without adrenalin, etc., were not employed. Massage of the heart would not have been employed had it not been found early in the course of the work that without it the heart started by stimulation would not develop a pressure that was sufficient to maintain the circulation.

Any one who has attempted to stop the heart by means of chloroform or asphyxia is aware of the fact that stoppage may result in several ways. It may be the result of inhibition, when it is sudden. More commonly it comes on gradually; the pulse becomes smaller and smaller, the blood pressure falls practically to zero, and soon, usually within a minute or two, the heart stops. Not infrequently, however, the heart continues to beat for many minutes, five, eight, or even more, after the pressure has fallen to its lowest level. The propelling power of the heart has practically ceased; the recording

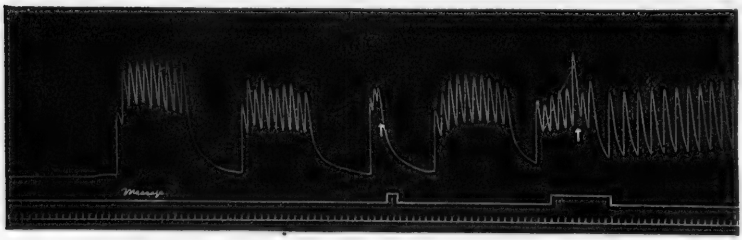
instrument may show only the faintest indication of a pulse. Occasionally the heart in this condition may recover under the influence of artificial respiration alone. On the other hand, it not infrequently happens that massage of the heart in addition to artificial respiration may fail to effect resuscitation. In such cases, as the protocols show, the beneficial effects of stimulation of the sinus region are especially striking.

In order to distinguish the beneficial effects of sinus stimulation from those of massage, the following procedure was usually followed. The heart was massaged as uniformly as possible several times, each time with the same number of strokes. In case this treatment revived the heart, the animal was again chloroformed or asphyxiated. In case it did not, approximately the same time interval was allowed to elapse between successive massages, and stimulation of the sinus region was combined with massage consisting of the same or of a smaller number of strokes.

It was discovered early in the course of the experiments that a wide range of strength of current could be employed for the purpose of making the auricles supply stimuli. A stimulus so strong as to cause the auricles to fibrillate has apparently been quite as effective as one not more than sufficient to start a regular beat. There was only this difference: the fibrillating auricle determines, as is the rule when conductivity is unimpaired, an irregular beat of the ventricles instead of a regular beat. In so far as the end results are concerned, however, the fibrillating and the regularly beating auricles are equally potent in that the arterial pressure maintained by the heart in delirium has been quite as high as that maintained by the heart at the moment it recovers from delirium (text-figure 1, *b*). For purposes of stimulation an inductorium was employed with five thousand windings in the secondary coil; it was actuated by one Edison-Lelande cell, type S. The distance between the secondary and the primary coils, as a rule, was between twenty and ten centimeters.

Not infrequently conductivity and irritability are lowered to such an extent by the cessation of the circulation that the impulses generated by an auricle that has been started by stimulation manifest no effect upon the ventricles. Under such circumstances mas-

sage and stimulation of the sinus have frequently caused the ventricles to beat with their inherent slow rate, although massage alone may have been without effect. I have no explanation to



1 2 3 4 5 6

TEXT-FIG. 1, *a*. Blood pressure in the carotid artery. Time in seconds. The signal indicates the level of zero pressure. (1) Massage. (2) Massage. (3) Massage plus stimulation; beat felt at point indicated by arrow. (4) Massage. (5) Massage plus stimulation; beat felt at point indicated by arrow. (6) Spontaneous ventricular contractions; the auricles are fibrillating.

offer of this beneficial effect of sinus stimulation upon the ventricles. The force of the ventricular beats so started has often been insufficient to maintain an effective circulation. As a rule, in such cases,

7 8

TEXT-FIG. 1, b. At 7 the impulses from the fibrillating auricles begin to affect the ventricles. At 8 the heart beat becomes normal.

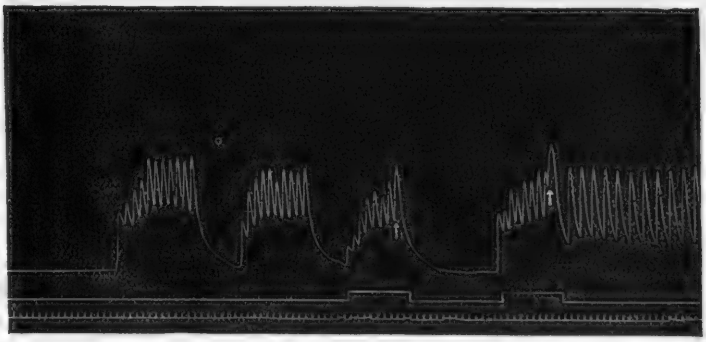
with the gradual improvement in the condition of the heart effected by the independent ventricles, the auricular impulses soon begin to reach the ventricles, with the result that the latter now beat more rapidly and effectively. In such cases, therefore, the beneficial

results of stimulation manifest themselves early, causing the ventricles to beat slowly, and also late, when the auricular impulses begin to effect the ventricles.

A large number of experiments have been performed; from these we select one typical experiment for the purpose of illustrating the foregoing discussion.

EXPERIMENT 4, SERIES II.

The stimulus was applied in this case to the inner wall of the right auricle, a stimulating sound having been inserted for this purpose through the jugular vein. Upon administration of chloroform, vagal effects appeared almost at once, and soon the respiration and the heart beat stopped. Massage given twice was negative (text-figure 2). Then stimulation was combined with massage. With



TEXT-FIG. 2.[5] Blood pressure in the carotid artery. Time in seconds. The signal indicates the level of zero pressure. (1) First period of massage. (2) Second period of massage. (3) Massage plus stimulation; beat felt at point indicated by arrow. (4) Massage plus stimulation; beat felt at point indicated by arrow. (5) Spontaneous ventricular contractions; the auricles are fibrillating.

the eighth compression of the heart a beat of the ventricles was perceived by the hand grasping the heart. After a pause, combined massage and stimulation were repeated. With the eighth compression another contraction of the ventricles was felt. Massage and stimulation were then stopped; the ventricles were seen to be beating slowly and regularly while the auricles seemed to be fibrillating. Suddenly the impulses of the fibrillating auricles began to affect the ventricles; at once the pressure, which had been practically stationary for some time, began to mount and soon reached its normal level. Later the fibrillations disappeared. Similar results were repeatedly obtained in this ex-

[5] All the text-figures have been reduced one half.

TEXT-FIG. 3. Blood pressure in the carotid artery. Time in seconds. The signal indicates the level of zero pressure. (1) The waves shown here are due to artificial respiration; the heart is not beating. (2) Massage. (3) Massage. (4) Massage plus stimulation followed by recovery of the heart.

periment (text-figure 1, *a* and *b*). Some of the trials show even more strikingly the beneficial effects of stimulation. For example, when the heart was stopped for the sixth time with chloroform the results were as follows: (*a*) massage (15 compressions), negative; (*b*) massage (10 compressions), negative; (*c*) massage combined with stimulation of the sinus; with the third compression a ventricular beat was felt under the hand; massage was immediately discontinued, but the heart did not beat further; (*d*) massage (10 compressions), negative; (*e*) massage plus stimulation; with the fifth compression the ventricles began to beat, while the auricles fibrillated. The ventricular beats were slow, strong, and regular until the impulses from the fibrillating auricles caused the ventricular beats to become irregular; thereupon results similar to those described above were again obtained. Later in this experiment (text-figure 3) the heart was recovered by massage plus stimulation but without fibrillation of the auricles.

EXPERIMENTS WITH THE CHEST UNOPENED.

After it had been demonstrated upon the heart freely exposed that stimulation of the sinus region is a valuable adjunct to massage in the resuscitation of the heart, the experiments were next directed toward devising a method of applying the principle in such a way as to make its use possible in the resuscitation of the heart of the ani-

mal with the chest unopened. Several procedures were considered: (1) The passage of currents through the heart either (*a*) by applying the electrodes externally, or (*b*) by applying one or both of the electrodes by way of the esophagus.[9] It was deemed inadvisable to employ either of these procedures, because of the difficulty of supplying currents of sufficient density at the desired place, and because of the risk consequently involved of exceeding the limit beyond which the ventricles might fall within the range of stimulation. (2) The use of a sound electrode applied to the sinus region through the jugular vein. The disadvantages of this method, which was also employed by Floresco for the application of a stimulus to the ventricles, are (*a*) the difficulty of bringing the electrode to bear against the sinus region of the auricles; (*b*) the time that is required to insert the sound into the vein; (*c*) the danger of passing the sound on into the ventricle; and (*d*) the danger of clotting. (3) The insertion of a sound electrode directly through the chest wall. A few trials convinced us that this method would be by far the

TEXT-FIG. 4. The stimulating sound electrode. A = steel tube; B = olive tip; C = insulated wire appearing at the surface through the insulated eye (D).

simplest and the least dangerous. A study of cross sections of the dog's chest indicated clearly the path that such a sound should be made to take, so as to reach the sinus region of the auricles.[10] The

[9] This approach has been used by Floresco (*loc. cit.*) for the stimulation of the whole heart.

[10] A fair idea as to the relations of the parts with which the sound comes into contact may be obtained from the cross sections of the dog's body published by Ellenberger and Baum, Anatomie des Hundes, Berlin, 1891. The plane in which the sound should lie is between those shown in plates VI and VII of their work.

sound finally developed is shown in text-figure 4 and requires no further description. It is inserted as follows: After nicking the skin in the third left interspace as close as possible to the sternal margin, the tip of the sound is placed against the subcutaneous tissue. It is directed perpendicularly to the chest wall and boldly pushed into the pleural space. The point is then directed to the right, and carried through the anterior mediastinum close to the sternum until it has passed some distance beyond the right border of the heart. The sound is then turned until the point looks downward and to the left, and while more and more of the sound is allowed to enter the chest, the outer end is carried upward and to the right. This pass will not meet with any resistance whatever, and will carry the point of the sound exactly to the sinus region of the heart. It just slides along the right side of the pericardium, or, in case it has been carried farther to the right, it is returned to the pericardium by slipping between the lobes of the lung from right to left. Unipolar stimulation is employed. The sound carries the stimulating electrode; the indifferent electrode is placed on the shaved thigh. The pericardium, it will be seen, lies between the electrode and the heart. Ordinarily it is so thin that it in no way interferes with the application of the stimulus to the heart. Occasionally, however, the pericardial fat is so abundant as to insulate the heart more or less completely. In such a case the method is of no value. In the course of our experiments this difficulty has been encountered only once. When the sound is withdrawn, even though no precautions are taken, air does not enter the chest, provided the subcutaneous tissue has not been cleaned away from the muscle. In practice, however, it might be well to take the precaution of laying a purse string ligature in the tissues about the sound, and of drawing it tight while withdrawing the sound. In this series of experiments stimulation of the sinus was employed as an adjuvant to subphrenic cardiac massage.

While the main object of this series of experiments has been to determine a feasible method of applying the tetanizing current to the sinus through the unopened chest, the experiments at the same time furnish additional indications of the value of the principle in the resuscitation of the heart. Brief extracts from the protocol of one experiment will suffice for purposes of illustration.

EXPERIMENT 12, SERIES III.

First Trial.—As a result of the administration of chloroform, respirations had ceased and the blood pressure had dropped to the lowest level. While the heart was still beating, artificial respiration was begun. Despite this the heart continued to grow weaker until its effect upon the record could scarcely be perceived. Ninety-three seconds after beginning artificial respiration, simple tetanic stimulation of the sinus did not improve the circulation. One hundred and sixty seconds later simple massage (20 strokes) effected only a very slight and transitory improvement. One hundred and five seconds after this, massage (20 strokes) combined with a short period of stimulation gave an immediate and perfect recovery.

Second Trial.—After the pressure had returned to normal, chloroform was again administered with results similar to those just mentioned. One hundred and seventy seconds after beginning artificial respiration, stimulation of the sinus region was negative. Ninety-five seconds later, massage (20 strokes) was negative. Text-figure 5 shows the terminal strokes of this period of massage. Thirty-two seconds later, massage (20 strokes) terminated by stimulation gave an immediate improvement. Two successive massages of 10 and 20 strokes each gave further slight improvement, while stimulation alone very materially hastened the recovery of the heart.

The third and fourth trials yielded results similar to those mentioned above.

TEXT-FIG. 5. Blood pressure in the carotid artery. Time in seconds. The signal indicates the level of zero pressure. (1) Termination of first period of massage. (2) Waves due to artificial respiration; the heart is beating, but the recording instrument shows no pulse. (3) Massage plus stimulation followed by improvement of the heart beat. (4) Massage followed by further slight improvement. (5) Massage followed by further slight improvement. (6) Stimulation followed by decided improvement.

RESUSCITATION OF THE ANIMAL.

In order to determine whether the method was suited not only to the temporary resuscitation of the heart and animal, but also to the permanent resuscitation of the apparently dead, in a few experiments the animals after resuscitation were permitted to recover

from the anesthesia. These experiments were performed under such aseptic precautions as it would be possible to employ in the case of an emergency. The animals were killed with chloroform administered in the usual way. Artificial respiration by the Meltzer and Auer method of tracheal insufflation was then started as quickly as possible. This often required several minutes. In some cases while this was being started an assistant made the incision into the abdomen through which subphrenic massage was to be given. In other experiments the abdomen was opened under ether anesthesia before the chloroform was administered. As soon as the artificial respiration had been started the stimulating sound was inserted. These preparations preliminary to beginning massage and stimulation usually required from four to six, or occasionally even ten minutes. Ten trials were made on as many animals. Three of these trials demonstrated clearly the value of stimulation as an accessory in permanent resuscitation. We do not mean by this to imply that stimulation was without effect in the other seven trials, but rather that in them the effects of stimulation were obscured by attending circumstances. Thus in some cases resuscitation was effected by artificial respiration or by artificial respiration and massage alone; that is to say, stimulation of the sinus proved to be unnecessary. In others accidents occurred which, it should be added, were almost invariably the result of inexperience rather than of any inherent defect in the method. In still others it happened that after successful resuscitation of the heart the respiratory center failed to become active. It is surprising how difficult it often is to stop the heart with chloroform. In the dog the respiratory center not infrequently recovers when the chloroform is withdrawn after respirations have ceased, and after the heart has become very weak. Indeed this may happen over and over again in the same animal, so that so much more chloroform has to be administered than is just necessary to stop the respirations that recovery of the tissues must become a much more difficult matter than in ordinary cases of chloroform poisoning. However this may be, it is obvious that the test to which the method is subjected in such experiments must be far more exacting than in usual cases of death from an overdose of chloroform on the operating table.

The protocols of two experiments will be given, one (experiment 9, series IV) for the purpose of illustrating the difficulties in the way of obtaining unambiguous results; the other (experiment 10, series IV), to illustrate a thoroughly successful resuscitation in which the value of stimulation of the sinus as an adjuvant to massage is clearly demonstrated.

EXPERIMENT 9, SERIES IV.

Time in minutes elapsing after beginning of chloroform administration.	Procedures and remarks.
0	Chloroform on.
½	Breathing stopped.
1½	Breathing begun.
1¾	Breathing stopped.
6	Heart very weak; artificial respiration.
6½	Massage.
7	Heart improved,[11] massage stopped.
9	Artificial respiration off.
9½	Spontaneous breathing.
10½	Chloroform on.
12	Breathing stopped.
13½	Heart slow and weak.
17	Artificial respiration on.
17¾	Heart beat scarcely palpable.
18	Heart stopped; massage.
18½	Heart beating; massage stopped.
19	Heart getting weaker.
19½	Massage.
20	Heart better; massage stopped.
22	Heart stronger; artificial respiration off.
24	No spontaneous respiration yet; heart weaker.
24	Artificial respiration on.
25	Heart very weak; massage.
26	Heart improving.
27	Massage off.
28	Artificial respiration off.
30	Spontaneous breathing.
31½	Heart weaker; breathing stopped.
32	Spontaneous breathing; heart improving.
36	Chloroform on.
38	Heart very weak.
38½	Heart stopped; massage.
40	Respiration stopped; artificial respiration and massage.

[11] The condition of the heart in this experiment was determined by palpation through the diaphragm either at the close of the periods of massage or during momentary interruptions of massage.

40½	Heart beating; artificial respiration and massage stopped.
41½	Heart weaker.
41¾	Artificial respiration on.
47	Heart very weak; massage.
47¾	Heart stopped.
48	Massage; heart beating shortly thereafter.
48½	Massage stopped.
49½	No improvement.
50	Heart weak.
50½	Massage plus stimulation.
51	Heart beating better; massage stopped.
53	Heart getting weaker.
53½	Massage.
54	Massage stopped; heart no better.
54½	Massage plus stimulation.
54¾	Heart better; massage continued.
56	No further improvement; massage continued.
57	Stimulation; massage continued.
58	Heart slow and no stronger; massage continued.
58½	Stimulation; massage continued.
59	Heart beating better; massage continued.
60	Beat slower; massage continued.
60½	Stimulation; massage continued.
62½	Heart beat fairly good; massage continued.
63½	Heart beat fairly good; massage continued.
67	Rectal temperature 31.5° C.
67	Artificial respiration off; massage stopped; heart continued to beat but the animal did not breath spontaneously; heart gradually weakened and stopped.

EXPERIMENT 10, SERIES IV.

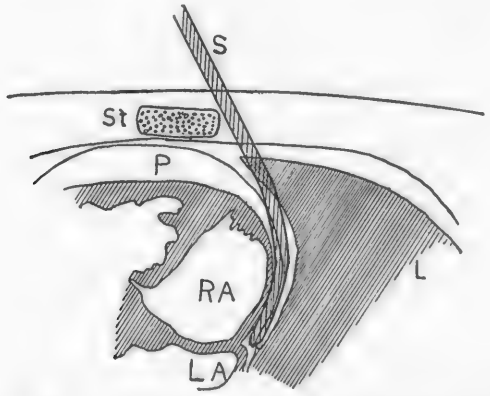
Time in minutes elapsing after beginning of chloroform administration.	Procedures and remarks.
0	Chloroform on.
1¼	Heart weaker.
2½	Breathing stopped.
3	Heart stopped.
3¾	Insufflation begun.
4	Massage.
4½	Heart not beating; massage continued.
5	Heart not beating; massage continued.
5¼	Heart not beating; massage continued.
6	Heart not beating; massage continued.
6½	Heart not beating; massage plus stimulation.
7	Heart not beating; massage continued.
7½	Heart not beating; massage plus stimulation.
9	Heart beating; massage continued.
10½	Heart beating; stimulation; massage continued.

11	Heart beating slowly but forcibly.
13½	Heart beat better but slow; massage stopped.
14	Artificial respiration off; spontaneous respiration begun at once. The wounds were closed aseptically, and the heart was beating strongly when the animal was taken from the table. Animal lived for seven days, and then died of intercurrent affection.

In this experiment insufflation was begun one and one fourth minutes after breathing had stopped, and massage of the heart one minute after the heart had stopped. Massage continued for two minutes failed to start the heart. The heart was first felt beating after the massage plus stimulation applied four and one half minutes after the heart had stopped.

APPLICABILITY OF THE METHOD TO THE RESUSCITATION OF MAN.

An examination of cross sections of the human body clearly indicates the feasibility of stimulation of the sinus region of the heart by the same method that has been employed in the case of the dog. Text-figure 6 shows the cross section of the body at the right level



TEXT-FIG. 6. Part of a cross section of the human body at the level of the second interspace. St = sternum; L = right lung; P = pericardial cavity; RA = right auricle; LA = left auricle; S = stimulating sound electrode.

for the introduction of the sound, with a sound of what would seem to be the proper shape in place. The opportunity has not been afforded to attempt the resuscitation of man by this method.

Accidents due to experimental manipulation have been very few. There is, of course, some danger of misdirecting the sound in such a way as to puncture the heart or lungs or to apply the stimulus to the ventricles instead of to the sinus region, thus causing the former to fibrillate. In our experiments the heart has never been punctured; the lungs were punctured once. The danger from this source is, therefore, very slight. And only once during the scores of times the current has been sent through the sound has fibrillation of the ventricles resulted. This danger cannot be great, since the sound when in the proper place can scarcely be brought into the vicinity of the ventricles; with practice in the passing of the sound one would soon learn to eliminate this danger entirely. A precaution that it might be well to take in order to obviate all danger of pneumothorax has already been referred to.

SUMMARY.

The four series of experiments described above indicate clearly that tetanic stimulation of the sinus region of the auricles is of material assistance to massage in the resuscitation of the heart. It causes the auricles to generate impulses, either rhythmical or irregular, to which the ventricles, when susceptible, respond. When massage administered for brief periods has failed to resuscitate the heart, the same period of massage combined with tetanic stimulation of the sinus has usually brought the circulation back to normal. In some instances massage alone when continued over long periods has been without effect, and to all appearances would have continued to be without effect, whereas at such a time massage combined with stimulation of the sinus has effected recovery. Whether the stimulus causes the auricles to beat normally or to fibrillate seems to make no essential difference in the end result. Not infrequently conductivity and irritability are lowered to such an extent by the cessation of the circulation, that impulses generated in an auricle started by stimulation can at first manifest no effect upon the ventricles. Under such circumstances massage alone or, more frequently, massage plus stimulation may cause the ventricles to beat with their inherent slow rate. Often then the force of the ventricular contractions is insufficient for the maintenance of an effec-

tive circulation. In such cases, however, with the gradual improvement in the condition of the heart, the auricular impulses, as a rule, soon begin to reach the ventricles, with the result that the latter immediately begin to beat more rapidly and effectively. The beneficial results of stimulation of the auricles, therefore, manifest themselves relatively late, but none the less effectively.

It has been found that even after the heart has begun to beat, stimulation of the sinus region may still be of some assistance, though in another direction. It not infrequently happens that after resuscitation of the heart the blood pressure fails to rise above a certain low and insufficient level. In such cases repeated tetanic stimulation of the sinus may accelerate the rise of blood pressure and so hasten recovery (protocol of experiment 12, series II, and text-figure 5). No attempt has been made to determine the mechanism of this effect.

THE ANTIGENIC PROPERTIES OF SPLIT PRODUCTS OF CASEIN.*

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Split products of proteins have been studied in their immunological relations from several points of view. Thus Obermeyer and Pick obtained by their studies with iodized and nitrated proteins interesting results on the physical nature of precipitinogens. Of particular interest have been the numerous though conflicting studies on the chemistry of anaphylaxis, designed either to show the relationship of the sensitizing to the intoxicating substance in various proteins (Rosenau and Anderson (1), Gay and Adler (2), Vaughan and Wheeler (3), Pick and Yamanouchi (4), Kraus and Doerr (5), Wells (6), and others), or else the nature of the toxic substance (Weichardt (7), Vaughan and Wheeler (3), Nicolle (8), Wells (6), and Schittenhelm and Weichardt (9)). Probably the greatest ultimate result that may be expected from a continuation of such studies will be some light on the essentially obscure problem of biological specificity.

With these points of interest in mind, particularly the latter, we have for some time been studying the antigenic properties of certain products derived from or obtained by combination with casein. Casein would seem to be a substance of particular interest for such investigation not only because it has been less systematically employed than other substances, such as serum, but because it is perhaps the only protein the chemical properties of which are thoroughly established.

ANAPHYLAXIS TO MILK, CASEIN, AND ITS DERIVATIVES.

Although milk anaphylaxis has been recognized since the pioneer work on the subject by Arthus (10) and Rosenau and Anderson

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(1), it has served rather for incidental than for serious investigation. The first attempt at a serious investigation by Besredka (11) is quite unconvincing owing to the crudeness of the chemical methods used in obtaining the various milk derivatives employed.

Our general scheme of experimentation has been to sensitize subcutaneously with skim-milk, casein, or split products of casein, normal guinea pigs of from two hundred and fifty to three hundred and fifty grams weight, and, after an incubation period of approximately three weeks, to attempt intoxication, usually by intraperitoneal injection, with one of the corresponding substances. A new Rosenau syringe, the cylinder of which had been thoroughly cleaned with bichromate of potash and sulphuric acid, and a new needle were used for each separate product, both for sensitizing and intoxicating, to avoid possible contamination with other proteins.

SERIES I.

Normal guinea pigs were sensitized by the injection of 1 c.c. of skim-milk that had been previously heated to 100° C. for fifteen minutes. The animals, after three weeks' interval, were given intraperitoneal injections of milk, casein (3 per cent. solution, corresponding to the amount present in milk), or a solution of glutamic acid plus leucin.

Preparation of Casein Solution.—The casein employed was Eimer and Amend's casein "nach Hammarsten," specially purified by the method of Robertson (12). This dried casein could be dissolved by physiological saline solution containing 8 c.c. of a $N/10$ potassium hydroxid solution in 100 c.c.

Preparation of Solution of Glutamic Acid and Leucin.—Kahlbaum's sub-

Guinea pig.	Sensitized.	Interval.	Second injection, intraperitoneal.	Symptoms.
7	1 c.c. milk	21 days	5 c.c. milk	Moderate.
8	1 c.c. milk	21 days	8 c.c. milk	Severe.
6	1 c.c. milk	21 days	8 c.c. 3 per cent. casein	Severe.
9	1 c.c. milk	27 days	8 c.c. glutamic acid + leucin	Negative; when given milk three days later showed severe symptoms.

stances were employed. Glutamic acid and leucin form the principal amino acids in casein from cow's milk and occur in the respective proportions of 11 and 10 per cent. (13). Our solution was prepared by dissolving these amino acids in alkaline salt solution so that the amount in 1.5 c.c. corresponded to 1 c.c. of milk (glutamic acid 0.22 per cent. + leucin 0.20 per cent. in 0.045 per cent. N sodium hydroxid + $M/6$ sodium chlorid 1.5 c.c. = 1 c.c. milk).

SERIES 2.

Guinea pigs were sensitized by 1 c.c. of 3 per cent. casein and intoxicated three weeks later by milk, casein, or paranuclein.

Preparation of Solution of Paranuclein.—Paranuclein is a product of the incomplete peptic hydrolysis of casein (Lubavin (14), and Kossel (15)). It was prepared in the following manner (Robertson and Biddle (16)). To a faintly acid solution of potassium caseinate (4 per cent.) was added 0.95 per cent. Grübler's pepsin *puriss. sicc.* and a little toluol. The mixture was then heated to 100° C. to destroy the pepsin; the paranuclein was filtered off, washed in a large volume of distilled water and in alcohol, and then dissolved in *N*/10 alkali and immediately precipitated with acetic acid. This precipitate was repeatedly washed in large volumes of distilled water, alcohol, and ether, and finally dried over sulphuric acid, pulverized, and sifted. The product was thus obtained in the form of a light, friable, white powder. The substance is probably a mixture of two or more different paranucleins (Robertson (17)), and its exact composition and power to neutralize bases probably varies somewhat with

Guinea pig.	Sensitized.	Interval.	Second injection, intraperitoneal.	Symptoms.
2	1 c.c. 3 per cent. casein	21 days	5 c.c. milk	Marked.
3	1 c.c. 3 per cent. casein	20 days	8 c.c. milk	Marked.
1	1 c.c. 3 per cent. casein	21 days	8 c.c. 3 per cent. casein	Severe.
4	1 c.c. 3 per cent. casein	23 days	5 c.c. 3 per cent. paranuclein	Marked.

the intensity of digestion (von Moraczewski (18)). It dissolves readily in dilute alkalis, neutralizing part of the alkali. One gm. of this paranuclein was found to neutralize 3.6 c.c. of *N*/10 potassium hydroxid with phenolphthalein as indicator.

SERIES 3.

Guinea pigs were sensitized to paranuclein.

Preparation of the Products of Peptic Digestion of Casein.—The solution of the products of the peptic hydrolysis of casein which was employed in these experiments was prepared as follows:

To 6 liters of *N*/50 sodium or potassium caseinate (containing 4 per cent. of casein) were added 2 gm. of Grübler's pepsin *puriss. sicc.*, which had previously been dissolved in a little water; this solution, after being thoroughly mixed, was allowed to stand at 36° C. for ten days; 2 gm. more of pepsin were added after the first four days, in the presence of toluol, and the solution was then sterilized by steam at 100° C. and filtered through hardened filter paper. To the filtrate were then added 2 gm. more of pepsin, dissolved, as in the previous cases, in a little water; toluol was introduced, and the solution was again allowed to stand at 36° C. for seven to eight days; it was then again sterilized by steam at 100° C. and filtered through hardened filter paper. The filtrate thus obtained is of a clear yellow color with little or no opalescence and gives no trace of a precipitate or opalescence upon the addition of acetic acid either before or after neutralization with alkali; hence both casein and paranucleins are completely absent from the solution.

Guinea pig.	Sensitized.	Interval.	Second injection.	Symptoms.
4a	5 c.c. 1 per cent. paranuclein	15 days	8 c.c. milk, intraperitoneally	Dead in 10 minutes. Immobilization of lungs; hemorrhages, lung and heart.
106	2 c.c. 1 per cent. paranuclein	23 days	5 c.c. milk, intraperitoneally	Very severe. Chloroformed in 1 hour. Extensive gastric hemorrhages.
117	1 c.c. 3 per cent. paranuclein	28 days	10 c.c. milk, intraperitoneally	Severe. Chloroformed in 3 hours. Gastric hemorrhages.
107	2 c.c. 1 per cent. paranuclein	23 days	8 c.c. 1 per cent. paranuclein, intraperitoneally	Severe.
103	2 c.c. 1 per cent. paranuclein	14 days	10 c.c. 1 per cent. paranuclein, intraperitoneally	Severe.
114	1 c.c. 3 per cent. paranuclein	28 days	1 c.c. 1 per cent. paranuclein, intravenously	Very severe.
110	2 c.c. 1 per cent. paranuclein	30 days	4 c.c. 12 per cent. peptic digestate, intraperitoneally	Severe.
115	1 c.c. 3 per cent. paranuclein	28 days	1 c.c. 6 per cent. peptic digestate, intravenously	Slight. No lesions.[1]
111	Control	———	4 c.c. 12 per cent. peptic digestate, intraperitoneally	Nearly as severe as in guinea pig 110.

SERIES 4.

Guinea pigs were sensitized with a solution of the products of peptic digestion of casein.

Guinea pig.	Sensitized.	Interval.	Second injection, intravenous.	Symptoms.
122	1 c.c. 3 per cent. solution	24 days	1 c.c. 1 per cent. paranuclein	No symptoms. Chloroformed. No lesions.
123	1 c.c. 3 per cent. solution	24 days	1 c.c. 6 per cent. solution peptic digestion	Slight agitation. Chloroformed. No lesions.
127	Control	———	1 c.c. 6 per cent. solution peptic digestion	Slight; as marked as in guinea pig 123.

SERIES 5.

Guinea pigs were sensitized with the amino acids corresponding to milk and serum. Glutamic acid 0.33 per cent in 0.45 per cent. *N* sodium hydroxid plus *M*/6 sodium chlorid. 1 c.c. corresponds to the amino acid as it occurs in casein. Glutamic acid plus leucin were as they occur in casein (series 1).

[1] See control guinea pig 127 in series 4.

Guinea pig.	Sensitized.	Interval.	Second injection, intraperitoneal.	Symptoms.
14	1.5 c.c. glutamic acid	15 days	5 c.c. glutamic acid	Negative.
13	1.5 c.c. glutamic acid	15 days	6 c.c. glutamic acid plus leucin	Negative.
15	1.5 c.c. glutamic acid	15 days	6 c.c. milk	Negative.
18	1.5 c.c. glutamic acid plus leucin	15 days	6 c.c. glutamic acid plus leucin	Negative.
20	1.5 c.c. glutamic acid plus leucin	15 days	7 c.c. milk	Negative.
21	6 c.c. glutamic acid plus leucin	15 days	6 c.c. milk	Negative.

The following conclusions may be drawn from these experiments on the antigenic power of split products of casein as tested by subsequent anaphylactic intoxication by the same products.

The amino acids (glutamic acid plus leucin; glutamic acid alone) in the proportions in which they occur in milk have no sensitizing property for subsequent intoxication by themselves or by milk.

The products of the peptic digestion of casein will not sensitize for subsequent intoxication by paranuclein. They have no more intoxicating property for guinea pigs sensitized by paranuclein or by peptic digestion products of casein than for normal animals.

Milk, chemically pure casein, and paranuclein sensitize to themselves and to one another and apparently indiscriminately in respect to the different doses employed in these experiments.

PROPERTIES OF ANTISERA TO CASEIN, PARANUCLEIN, AND AMINO ACIDS.

Rabbits were immunized by repeated injections of glutamic acid, glutamic acid plus leucin, paranuclein, and casein, and at a proper period subsequent to the last injection were bled and the respective sera removed, inactivated at 56° C., and subsequently tested for precipitins and fixation antibodies.

The protocols of these animals may be briefly summarized as follows:

Rabbit 1 was given intravenous injections of a solution of glutamic acid plus leucin in doses of 1.5, 2, and 1.5 c.c. at intervals of four days. The animal was bled nine days later.

Rabbit 2 was given intravenous injections of glutamic acid solution in doses of 1.5, 3, and 2 c.c. at intervals of four days. The animal was bled nine days later.

Rabbit 101 was given four intraperitoneal injections of 10 c.c. of a 2 per cent. solution of casein at intervals of seven days. The animal was bled seven days later.

Rabbit 100 was given four intraperitoneal injections of a 2 per cent. solution of paranuclein at intervals of seven days. The animal was bled seven days later.

Rabbit 104 was given three intraperitoneal injections of a 2 per cent. solution of paranuclein at intervals of four days. The animal was bled nine days later.

PRECIPITIN EXPERIMENTS.

The sera of rabbits 1 and 2 tested in a dose of 0.5 of a cubic centimeter with doses of filtered milk, ranging from 0.05 to 0.4 of a cubic centimeter, and of glutamic acid plus leucin solution, 0.1 to 1.2 of a cubic centimeter, showed no evidence of precipitin formation and were not investigated further. The antisera to casein (rabbit 101) and to paranuclein (rabbits 100 and 104) were tested more systematically, as they were found to give positive results. Anticasein serum (rabbit 101) was tested several times with 1 per cent. solution of casein, paranuclein, and once with a 1 per cent. solution of the products of peptic digestion. The solutions of casein and paranuclein were prepared in saline solution containing sufficient $N/10$ potassium hydroxid (casein eight to one hundred cubic centimeters; paranuclein 3.6 to one hundred cubic centimeters). The smallest amounts of such solutions that sufficed to give a distinct precipitin sedimentation with 0.3 of a cubic centimeter of the antiserum were as follows:

Casein, 1 per cent. solution.....Positive, 0.001 to 0.0001 c.c.
Paranuclein, 1 per cent. solution.....Positive, 0.01 c.c.
Peptic digestion, 1 per cent. solution.....Negative, 0.01 c.c.

The antiparanuclein sera (rabbits 100 and 104) gave no positive precipitin reactions with casein, paranuclein, or peptic digestion products in amounts as high as 0.3 of a cubic centimeter of a 1 per cent. solution.

FIXATION EXPERIMENTS.

As might be anticipated, the fixation reaction was found a far more delicate test for antibodies than the precipitin reaction. The tests were carried through in a similar manner to the precipitin reactions and usually synchronously. The details of the technique may be summarized as follows:

Antigenic Mixtures.—1 per cent. solution in saline with a total volume of 1 c.c.

Antiserum.—0.3 c.c. (56° C.).

Alexin.—0.1 c.c. of mixed serum from several guinea pigs removed from the clot eighteen to twenty-four hours after bleeding. Dilution to 1 c.c. in salt solution. Incubating with antigen and antibody at 37° C. for 1 hour.

Hemolytic System.—1 c.c. of a 5 per cent. suspension of washed sheep blood containing four minimal hemolytic doses of a strong rabbit antisheep serum.

Positive Fixation.—Complete absence of hemolysis after two hours at 37° C. followed by sedimentation overnight in the ice box.

Controls.—Each antigenic dilution plus 0.3 c.c. of inactivated normal rabbit serum. Antiserum with salt solution replacing antigen. These should hemolyze completely.

The reactions with anticasein serum (rabbit 101) gave complete fixations as follows:

Casein, 1 per cent. solution.....0.000,001 to 0.000,000,1 c.c.
Paranuclein, 1 per cent. solution.....0.001 c.c.
Peptic digestion, 1 per cent. solution.....Negative, 0.1 c.c.

Antiparanuclein serum (rabbit 104) gave no fixation with 0.1 of a cubic centimeter of the 1 per cent. solutions of casein, paranuclein, or peptic digestion products. It may be noted that this dose represents the largest amount that could be used for the reaction; with normal serum in larger doses distinct inhibition of alexin in the controls is produced. Serum 100, obtained in precisely the same manner as the potent anticasein serum 101, but by injecting paranuclein, had only weak fixing power for paranuclein (0.01 of a 1 per cent. solution) and produced no fixation with casein or with the products of peptic digestion of casein.

SUMMARY.

We draw the following conclusions from our experiments on the antigenic properties of chemically pure casein and some of its split products.

Casein and paranuclein have distinct antigenic properties, particularly as shown by their ability to sensitize guinea pigs for subsequent anaphylactic intoxication by each other or by milk. This sensitizing ability and a corresponding ability to intoxicate are indistinguishably equivalent, under the conditions employed. On immunizing rabbits by repeated injections of paranuclein or of

casein, and subsequently testing their sera for precipitins and fixation antibodies, it was found that casein apparently produces them much more readily, giving an antiserum that reacted (fixation) in very high dilution with casein (0.000,000,1 of a 1 per cent. solution), but much less strongly with paranuclein. Only one of two antiparanuclein sera showed the presence of antibodies to paranuclein by the delicate fixation reaction, and that in relatively small amounts. The two antibodies to casein and to paranuclein are, in the case of casein quantitatively, and in the case of paranuclein absolutely specific.

A solution of the products of complete peptic digestion of casein fails to sensitize to paranuclein and gives no fixation reaction with an anticasein or antiparanuclein serum. It intoxicates animals sensitized to paranuclein but no more markedly than it does normal animals. It also fails to show specific intoxication in an animal that has been sensitized by the same substance.

The amino acids, glutamic acid, and leucin, the principal components of their kind in casein, and in the same proportion therein present, likewise failed to show antigenic properties. They do not sensitize animals to milk intoxication or to intoxication by themselves, and likewise failed to produce precipitins in rabbits in a preliminary experiment.

These experiments are regarded as a fairly systematic analysis of the antigenic properties of split products of a single protein. They are analogous to, though less complete than the work of Wells (6) on egg-white. They seem to present the additional advantage of dealing with what is probably the only protein certainly known chemically, and in its purest form. They serve, moreover, as an introduction to the following study of the antigenic properties of a combined protein.

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THE ANTIGENIC PROPERTIES OF A PROTEIN COMPOUNDED WITH CASEIN.*

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One of the most promising methods of attack on the obscure problem of biological specificity would seem to lie in the search for newly formed antigenic properties in proteins compounded either of non-antigenic proteins, or of an antigenic with a non-antigenic protein. It was with this method in mind that we sought for possible antigenic properties in the mixture of the amino acids (glutamic acid plus leucin) in the proportion in which they occur in casein (1). No success was obtained in this case, however. We have since studied in more detail the antigenic properties of protamin (salmin) as compared with a compound substance that it forms with casein.

The preparation of the pure casein that we have employed and the properties of a potent antiserum to it have been discussed in the previous article. The methods of preparing protamin and protamin caseinate are as follows:

Protamin (*Salmin*).—A member of the protamin group, namely, salmin (according to Taylor (2), the protamin which is contained in the sperm of the Pacific salmon is identical with the salmin found in the sperm of the European salmon), was prepared by the method of Kossel (3) as follows:

The ripe testicles of the Pacific salmon were minced and the macerated mass which was thus obtained was shaken up in tall glass cylinders with five or six times its volume of distilled water. The thick suspension of sperm which was thus obtained was syphoned off from the subnatant connective tissue and curdled by the addition of 80 c.c. per liter of *N*/10 acetic acid. The curdled mass of sperm was then washed in ten times its volume of 95 per cent. alcohol; this washing was repeated twice; it was then washed once in the same volume of absolute alcohol and then in the same volume of ether. The powder, wet with ether, was spread out upon filter paper to dry in the air in a warm dry place.

Each 15 gm. of the dried sperm was then stirred up in 350 c.c. of 1 per cent.

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sulphuric acid (by volume) for about six hours. This mixture was then filtered and the filtrate obtained from the extraction of 15 gm. of sperm was placed in a tall glass cylinder of about 4,000 c.c. capacity which was then filled with absolute alcohol. After allowing the precipitate to settle, the supernatant fluid was syphoned off, the precipitate contained in two cylinders was collected in one, and this was again filled with alcohol. The entire precipitate, suspended in alcohol, from the extract of 300 gm. of sperm, was dissolved by the addition of about 4 liters of hot water (about 80° C.); the least soluble portion was filtered off, and the remainder reprecipitated by the addition of 10 volumes of alcohol. This precipitate was washed once in the same volume of alcohol as that employed in precipitation and then in a like volume of ether. The final suspension of salmin sulphate in ether, obtained after syphoning off the supernatant ether, was collected on a hardened filter, dried over sulphuric acid at 40° C. for two days and then pulverized and sifted. The product is a friable, white powder.

Protamin Caseinate.—It was pointed out by Kossel (4) that when members of the protamin group are mixed with other proteins in weakly alkaline solutions precipitates are produced. Kossel suggested that the protamins combine with other proteins to form compounds analogous to the naturally occurring histons. Hunter (5) has further investigated these precipitates and finds that crystalline egg-albumin, casein, hemielastin, gelatin, edestin, hetero-albumose, protalbumose, alkali-albuminate, and histon sulphate form compounds with protamins which are insoluble in ammoniacal solutions. According to Hunter, the compound of casein with clupein contains 1 part in 3.5 of clupein.

Many attempts were made to obtain salmin caseinate in quantity with but poor success. It was not until nearly all immediately available supplies of salmin were used up that it was found that one of the most important sources of difficulty lay in the fact that salmin caseinate is soluble, or at least forms a permanent suspension, in the presence of excess of ammonium caseinate. Finally, a very small amount of a substance that was presumably salmin caseinate was prepared in the following manner:

2.4 gm. of salmin sulphate were dissolved in 200 c.c. of *N*/10 ammonia and 5 gm. of casein were dissolved in 200 c.c. of water containing 150 c.c. of *N*/10 ammonia. The two solutions were filtered and then mixed and diluted to 3,600 c.c. A very fine granular precipitate formed very slowly and settled, leaving the supernatant fluid distinctly opalescent. This precipitate was collected upon a hardened filter, washed in large volumes of alcohol and ether, (ammonium caseinate is soluble in alcohol containing traces of water (Robertson (6)), so that any adherent casein would be removed by this procedure) and dried over sulphuric acid. The yield was only 0.7 gm. The substance was insoluble, only a portion dissolving even in *N*/10 alkali. An approximate determination of the nitrogen content made upon a very small sample (50 mg.) yielded the result N = 15.8 per cent., practically the nitrogen content of pure casein. The quantity obtained was insufficient to carry out any satisfactory analyses for the purpose of determining the relative proportion of salmin and casein in the compound.

Taylor (7) has already noted that protamin obtained by the method described is toxic for rabbits and has also mentioned his

failure to produce an antiserum to the substance. His method of detecting antibodies was crude (visible cytolysis), and no details of experimentation are given. We have found it difficult to carry out anything approaching an immunization process in rabbits, owing to the toxicity of the protamin and the fact that successive doses show no evidence of increased tolerance. Thus of three rabbits that were given successive subcutaneous or intraperitoneal injections of five cubic centimeters of a 2 per cent. solution of protamin in salt solution, only one survived four injections given at intervals of four or five days. Symptoms of collapse with increased respiration and tachycardia follow the injections by either route. Recovery is apparently complete in an hour or two, but the animals lose weight rapidly and die four or five days later with no obvious lesions. Rabbit 50 withstood four injections of five cubic centimeters of a 2 per cent. solution given at intervals of five days and was bled eight days later and the serum inactivated for the following tests.

Our preparation of protamin caseinate differs markedly from protamin in being absolutely non-toxic for rabbits even when injected intravenously. The preparation goes only partially into solution even in $N/10$ potassium hydroxid, but in the case of intraperitoneal or subcutaneous injections for immunization, the insoluble residue as well as the supernatant solution was injected. Thus rabbit 54 was given two cubic centimeters of a 1 per cent. protamin caseinate solution intravenously, followed by two subcutaneous injections of ten and five cubic centimeters each, at intervals of eight and five days respectively. These injections were followed by two more intraperitoneal injections ten and thirteen days later of thirteen and ten cubic centimeters each. The animal was bled to death eight days later.

It need scarcely be noted that the greatest care was exercised in obtaining chemical cleanliness in these experiments. A separate new Rosenau syringe was kept for injections of each of these substances, as in the experiments recorded in the previous article.

Fixation experiments were then carried out with sera of these two treated animals (50 and 54) as well as with the anticasein serum animal (101) that has been already described. The technical details and controls of these experiments are precisely the same as

in the previous article. The following table summarizes several experiments that have been made in cross fixation between protamin, protamin caseinate, and casein, and their corresponding antisera.

TABLE I.

Fixation Reactions between Protamin, Protamin Caseinate, and Casein, and the Sera of Animals Repeatedly Injected with Each of Them.

Antigenic solution 1 per cent. in volume of 1 c.c.	Antiserum to protamin (rabbit 50). 56° C., 0.3 c.c.	Antiserum to protamin caseinate (rabbit 54). 56° C., 0.3 c.c.	Antiserum to casein (rabbit 101). 56° C., 0.3 c.c.
Protamin	Negative 0.1 c.c.	Negative 0.1 c.c.	Negative 0.1 c.c.
Protamin caseinate	Negative 0.1 c.c.	Positive 0.0001 c.c.	+ Positive 0.001 c.c.
Casein		Positive 0.0001 c.c.	Positive 0.00001 c.c.

It is evident from these results that protamin does not act as an antigen in that it does not react with the serum of a rabbit that has received repeated injections of it or of protamin caseinate. Protamin caseinate is antigenic in virtue of its casein content, reacting almost equally well both with an antiserum to itself and to casein. The antiserum to casein conversely gives a good fixation with protamin caseinate. It is evident, therefore, that these experiments with a compound of casein and a non-antigenic protein (protamin caseinate) throw no light on the origin of specificity.

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STUDIES ON FERMENT ACTION.

III. A Specific Immune Lipase.*

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It has been shown apparently, within the last few years, that antigenic agents are not limited to the albuminous compounds, but that certain lipoid substances have the property of stimulating antibody formation within a treated host. Thus, Much (1) has observed that nastin, a fatty substance obtained from acid-fast bacteria, gives specific reactions, both local and general, in individuals suffering with diseases caused by this class of microörganisms. He was also able to get positive complement fixation tests with the sera of these patients, using nastin as antigen. Kurt Meyer (2) has been successful in getting complement deviations with the sera of patients infected with animal parasites, using as antigen alcoholic extracts from these organisms. He also obtained complement fixation tests with the sera of animals artificially infected with these parasites. Citron and Reicher (3), Peritz (4), and others have demonstrated that the lipase content of the sera of luetics is considerably increased. The same is true of patients afflicted with leprosy, tuberculosis, and other diseases, the causative organisms of which have fatty or lipoid constituents. Gottlieb and Lefmann (5) were able to prepare specific antibodies (hemolysins) by treating animals with ethereal extracts of red blood corpuscles. Bergel (6) has recently demonstrated that after successive intraperitoneal and and intrapleural injections of lecithin the lipase content of extracts from the spleen, lymph-glands, liver, and blood serum is considerably increased. Furthermore, positive complement fixation was obtained with these extracts and sera, the sera proving much weaker than the extracts. Consequently, we may consider the antigenic properties of lipoids as well established.

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If we approach the question at hand from another point of view, it is of interest to note that Neuberg and Reicher (7), Neuberg and Rosenberg (8), and others have demonstrated a marked parallelism between agglutinative and hemolytic, and lipolytic substances. These agents employed had, moreover, varied origins; namely, animal, vegetable, and bacterial. Among the most carefully studied of these substances we find abrin, crotin, ricin, cobra venom, bee poison, the fat-splitting ferments of the gastric and pancreatic juices, staphylolysin, procyanolysin. This parallelism is so strict that one can classify hemolytic agents and put them in order of activity by a knowledge of their lipolytic properties. This relation is so impressive that more than one experimenter (Neuberg and Reicher, Bergel, and others) has considered whether hemolysis is a kind of lipolysis, or, at least, whether the lipolytic activities of a hemolysin play a direct and essential part in its functioning.

On the one hand are seen the antigenic powers of lipoids, ethereal extracts of red blood corpuscles, etc.; on the other appears the intimate relation of lipolytic and hemolytic substances. Further, it is noted that the serolipolytic activities of animals treated with lecithin are increased for alien fat, and that immunization with foreign erythrocytes will produce the same phenomenon. Hence arise the questions as to the specificity of these reactions and the particular rôle played in hemolytic processes by the different agents. It is to assist in answering these questions that the experiments dealing especially with the lipolytic properties of specific hemolytic sera have been undertaken. A consideration of the particular part played by these lipolytic agents in serum hemolysis and other immune processes will be reserved for future study and separate publication.

In Bergel's (6) recent publications it is stated that the sera of animals immunized to foreign red blood cells are about twice as active in splitting foreign fats as the sera of untreated animals. The results were partially confirmed by our own work (table I). The discrepancy that we find may be due, in part, to the method employed in immunizing the animals and to other points of technique. However this may be, the increase in serum lipase described by him is not in any way shown to be specific; the digestion experiments

were made with only such substances as lecithin (Merck), ethylbutyrate, butter fats, etc.; that is, all alien fats or lipoids. Therefore, the true significance of this increased lipolytic activity was not suspected or determined.

In our work all animals (rabbits) were immunized and all lipolytic sera were obtained by giving a subcutaneous injection of the washed corpuscles from 7 c.c. of hen's blood, and five days later an intravenous injection of the corpuscles from 10 c.c. of blood prepared in a similar way. From five to ten days after the last injection the rabbit serum had a high hemolytic titer. The titrations were made with *N*/100 sodium hydroxid from a 2 c.c. burette and the results recorded in the number of cubic centimeters of *N*/10 sodium hydroxid necessary to neutralize 100 c.c. of the titrated solution. This method of titrating and recording was especially convenient since we were dealing, as a rule, with small quantities and wished our results in a form convenient for purposes of comparison.

Our first endeavor was to secure an accurate basis of comparison between the non-specific lipolytic activity of the serum of animals before and after treatment with foreign red blood cells. Since the lipase content of the sera of different individuals of the same species is variable, it was necessary to use a given animal throughout the course of the experiments. Therefore, the sera of normal rabbits were tested for their activity against ethylbutyrate. When the sera of the same rabbits, following injections of foreign corpuscles, had reached a high hemolytic titer, they were again examined in a similar manner.

For this purpose, 25 c.c. of 0.85 per cent. sodium chlorid solution were put in each of five Erlenmeyer flasks. After the addition of 0.5 c.c. of tuluol and 0.1 c.c. of a 1 per cent. alcoholic solution of phenolphthalein, the solutions were brought to the boiling point to drive off the carbon dioxid. When the flasks had cooled to 37° C., the desired amount of serum was added to each of the flasks. Number 5, to which no serum was added, was used as a control. The mixtures were then neutralized with *N*/50 sodium hydroxid; 1 c.c. of acid-free ethylbutyrate was put in each, shaken one hundred times to get a homogeneous emulsion, and incubated at 37° C. for four hours. At the end of this time, all flasks were immediately cooled to 5° C. and titrated with *N*/100 sodium hydroxid. The results obtained with the normal and immune serum are recorded in the (a) and (b) divisions of table I, respectively

TABLE I.

No.	0.85 per cent. sodium chlorid.	Fresh normal rabbit serum.[1]	Acid-free ethyl-butyrate.	Acidity.
1a	25 c.c.	1.0 c.c.	1 c.c.	1.4 c.c.
2a	25 c.c.	0.2 c.c.	1 c.c.	0.7 c.c.
3a	25 c.c.	0.1 c.c.	1 c.c.	0.5 c.c.
4a	25 c.c.	0.01 c.c.	1 c.c.	0.3 c.c.
5a	25 c.c.	1.0 c.c. 0.85 per cent. sodium chlorid	1 c.c.	0.2 c.c.
1b	25 c.c.	1.0 c.c.	1 c.c.	1.9 c.c.
2b	25 c.c.	0.2 c.c.	1 c.c.	0.8 c.c.
3b	25 c.c.	0.1 c.c.	1 c.c.	0.5 c.c.
4b	25 c.c.	0.01 c.c.	1 c.c.	0.3 c.c.
5b	25 c.c.	1.0 c.c. 0.85 per cent. sodium chlorid	1 c.c.	0.2 c.c.

By observing the corresponding numbers of the (a) and (b) divisions of table I, normal and immune serum respectively, it will be seen that the immune hemolytic serum in the various amounts used is only slightly more active upon ethylbutyrate (foreign fat) than the serum of the same rabbit before immunization, but at the same time this increase is quite noticeable. The various dilutions were used for comparison with a later table, to which reference will be made.

Having secured the relative values of the non-specific lipolytic properties of the normal and immune serum, our next efforts were set to determine, if possible, whether this lipase content is in any way specific; *i. e.,* whether the lipoids or lipoid combinations peculiar to the specific cells were especially susceptible to the action of the above mentioned lipase. In the light of the work of Bang and Forssman (9), a positive result here seemed probable. These authors found that only a certain lipoid fraction of the ethereal extract of erythrocytes was active in producing a specific immune hemolytic serum, the albuminous portions being definitely excluded. If an antigen is lipoidal in nature, what will be the chemical nature of the antibody formed in reaction to its presence in a host? An agent which will aid the animal in an appropriation or

[1] In the course of these experiments it was observed that inactivation of serum at 56° C. for twenty minutes reduces very materially its lipase activity. This activity is also reduced by age inactivation, but not so much as by the heat method. So, for comparative purposes, it is very necessary to use all sera in the fresh or inactivated condition and of the same age.

an elimination of the alien substance is the most reasonable thing to expect; that is, a lipoidal foreign substance, a lipase reaction product. Our efforts here were rewarded with positive results, as the following experiments will show. The specificity can be shown, however, only by observing the lipolytic effects of an immune serum upon the specific corpuscles and by a comparison of these with the results obtained when various other corpuscles (human, rabbit, guinea pig) and normal sera are substituted for the specific corpuscles and serum, respectively.

Five c.c. of a 5 per cent. suspension of washed hen corpuscles were incubated for two hours with definite amounts of an immune hemolytic serum, of normal rabbit serum, and guinea pig serum, and the acidity of the resultant fluids was titrated. The immune serum used had a hemolytic titer of 0.0007 c.c. Both normal and immune sera were inactivated at 56° C. for twenty minutes and used in the tests as indicated. At the end of the incubation period, all mixtures were brought up to the boiling point in order to drive off the carbon dioxid, which would otherwise interfere with the titrations. The boiling also served to reduce the hemoglobin, where hemolysis had occurred, giving a clear or opalescent fluid which can be accurately titrated with phenolphthalein as an indicator. Twenty drops of a 1 per cent. alcoholic solution of phenolpthalein were added to 75 c.c. of 0.85 per cent. sodium chlorid solution, the mixture was boiled, and 1 c.c. added to each 2 c.c. of the solution to be tested. The titrations were made with *N*/100 sodium hydroxid from a 2 c.c. burette and recorded in the number of cubic centimeters of *N*/10 sodium hydroxid necessary to neutralize 100 c.c. of the solutions. The results appear in table II.

TABLE II.

No.	Hen corpuscles, 5 per cent. suspension.	Inactivated serum.[2]	Guinea pig complement.	Incubation time.	Acidity.
1	5 c.c.	0.1 c.c. I.H.S.	0.04 c.c.	2 hrs.	1.8 c.c.
2	5 c.c.	0.1 c.c. N.R.S.	0.04 c.c.	2 hrs.	0.6 c.c.
3	5 c.c.	0.1 c.c. I.H.S.	———	2 hrs.	0.9 c.c.
4	5 c.c.	0.1 c.c. N.R.S.	———	2 hrs.	0.4 c.c.
5	5 c.c.	———	0.04 c.c.	2 hrs.	0.6 c.c.
6	5 c.c.	———	———	2 hrs.	0.4 c.c.
7	5 c.c. 0.85 per cent. sodium chlorid	0.1 c.c. I.H.S.	0.04 c.c.	2 hrs.	0.1 c.c.
8	5 c.c. 0.85 per cent. sodium chlorid	0.1 c.c. N.R.S.	0.04 c.c.	2 hrs.	0.1 c.c.
9	5 c.c. 0.85 per cent. sodium chlorid	———	0.04 c.c.	2 hrs.	0.0 c.c.
10	5 c.c. 0.85 per cent. sodium chlorid	0.1 c.c. I.H.S.	———	2 hrs.	0.1 c.c.
11	5 c.c. 0.85 per cent. sodium chlorid	0.1 c.c. N.R.S.	———	2 hrs.	0.1 c.c.

[2] I.H.S. = immune hemolytic serum; N.R.S. = normal rabbit serum.

In this experiment (table II) ten hemolytic units of immune serum for each unit of corpuscles and a corresponding quantity of normal serum were used, the complement being reduced to an amount just sufficient to cause hemolysis by the hemolytic serum, for the purpose of eliminating as much as possible the lipolytic action of the guinea pig serum.

By observing the numbers 1 and 2 of the acidity column of table II, we see that the immune serum, specific corpuscles, and complement give three times the acidity produced when normal serum is substituted for the immune. In numbers 3 and 4, where no complement was used, the immune mixture, number 3, is more than twice as acid as number 4, normal serum. The other numbers, including a complement corpuscle mixture, corpuscles alone, and combinations of the complement, immune, and normal sera in a physiological salt solution show that the acidity is largely due to the action of the sera upon the corpuscles.

The difference between the acidities produced by the normal and immune sera when a corpuscular suspension is used as a substrate is much greater than that obtained when ethylbutyrate is used. To decide the question as to whether this same difference would obtain when various foreign corpuscles are used instead of the specific ones, corpuscular suspensions of hen (specific), human, rabbit, and guinea pig blood were collected and prepared in the usual way and digested with the immune serum used in the previous work, both with and without complement. The results recorded in table III show, by comparison of the (a), (b), (c), and (d) divisions of the protocol, that the acidity of the immune serum and specific erythrocyte mixture (part (a)) is three times as great as that of either of the other mixtures. If normal serum and specific corpuscles, and the specific serum, and various foreign corpuscles fail to produce the acidity given by an action of the immune serum upon the specific corpuscles, the last named corpuscles must be especially susceptible to the action of the immune serum.

As shown by the numbers 3 and 4 of table II and 1a of table III, the presence of complement is not necessary in order to distinguish the lipase activity of immune and normal sera when the specific corpuscles are used as substrate. For the purpose of emphasizing

TABLE III.

No.	5 per cent. suspension of corpuscles.	Antihen serum.	Guinea pig serum.	Incubation time.	Acidity.
1a	2 c.c. (hen)	0.004 c.c.	0.16 c.c.	2 hrs.	1.6 c.c.
2a	2 c.c. (hen)	0.004 c.c.	———	2 hrs.	0.8 c.c.
3a	2 c.c. (hen)	———	0.16 c.c.	2 hrs.	0.4 c.c.
1b	2 c.c. (human)	0.004 c.c.	0.16 c.c.	2 hrs.	0.5 c.c.
2b	2 c.c. (human)	0.004 c.c.	———	2 hrs.	0.5 c.c.
3b	2 c.c. (human)	———	0.16 c.c.	2 hrs.	0.4 c.c.
1c	2 c.c. (guinea pig)	0.004 c.c.	0.16 c.c.	2 hrs.	0.5 c.c.
2c	2 c.c. (guinea pig)	0.004 c.c.	———	2 hrs.	0.4 c.c.
3c	2 c.c. (guinea pig)	———	0.16 c.c.	2 hrs.	0.3 c.c.
1d	2 c.c. (rabbit)	0.004 c.c.	0.16 c.c.	2 hrs.	0.5 c.c.
2d	2 c.c. (rabbit)	0.004 c.c.	———	2 hrs.	0.5 c.c.
3d	2 c.c. (rabbit)	———	0.16 c.c.	2 hrs.	0.3 c.c.
Salt control	2 c.c. (hen)	———	———	2 hrs.	0.2 c.c.
Salt control	2 c.c. (human)	———	———	2 hrs.	0.3 c.c.
Salt control	2 c.c. (guinea pig)	———	———	2 hrs.	0.2 c.c.
Salt control	2 c.c. (rabbit)	———	———	2 hrs.	0.2 c.c.

this point, a special protocol (table IV) was prepared. The elements used in this table were prepared in the same way as the corresponding elements of the previous tables, and the results were obtained and recorded as usual, so that a detailed description is not thought necessary. The difference between the acidities produced by the normal and immune sera (table III) is not as great where no complement is used, but a noticeable difference still

TABLE IV.

No.	Hen corpuscles, 5 per cent. suspension.	Serum.[3]	Incubation time.	Acidity (amount N/10 sodium hydroxid to neutralize 100 c.c.
1a	5 c.c.	0.1 c.c. S.Im.S.	4 hrs.	2.0 c.c.
2a	5 c.c.	0.05 c.c. S.Im.S.	4 hrs.	1.2 c.c.
3a	5 c.c.	0.02 c.c. S.Im.S.	4 hrs.	0.9 c.c.
4a	5 c.c.	0.01 c.c. S.Im.S.	4 hrs.	0.8 c.c.
5a	5 c.c.	0.001 c.c. S.Im.S.	4 hrs.	0.6 c.c.
1b	5 c.c.	0.1 c.c. N.R.S.	4 hrs.	1.0 c.c.
2b	5 c.c.	0.05 c.c. N.R.S.	4 hrs.	0.6 c.c.
3b	5 c.c.	0.02 c.c. N.R.S.	4 hrs.	0.5 c.c.
4b	5 c.c.	0.01 c.c. N.R.S.	4 hrs.	0.5 c.c.
5b	5 c.c.	0.001 c.c. N.R.S.	4 hrs.	0.3 c.c.
Control	5 c.c.	0.1 c.c. 0.85 per cent. sodium chlorid	4 hrs.	0.3 c.c.

[3] S.Im.S. = specific immune serum; N.R.S. = normal rabbit serum.

remains. This shows that this immune lipase acts more or less independently of complement. The rapidity of acid production, however, is much greater in the presence of complement. Hence a four hour incubation period was used for this protocol.

In table I, where the ferment action of the normal and immune sera against an alien fat (ethylbutyrate) was compared, we observed that the acidities produced by the corresponding quantities of sera were only slightly different, the immune serum being somewhat more active than normally. Here the acidity produced by a certain amount of immune serum is obtained with the normal serum by using less than twice that amount. If the specific corpuscles are used as substrate, we may ask what multiple of normal serum will be necessary to give the acidity produced by a certain amount of the immune serum. In table V, in which 0.1 of a cubic centimeter of

TABLE V.

No.	Hen corpuscles, 5 per cent. suspension.	Serum.	Complement.	Incubation time.	Acidity.
1	5 c.c.	0.1 c.c. S.Im.S.	0.04 c.c.	2 hrs.	1.4 N/10 sodium hydroxid
2	5 c.c.	0.1 c.c. N.R.S.	0.04 c.c.	2 hrs.	0.4 N/10 sodium hydroxid
3	5 c.c.	0.2 c.c. N.R.S.	0.04 c.c.	2 hrs.	0.6 N/10 sodium hydroxid
4	5 c.c.	0.3 c.c. N.R.S.	0.04 c.c.	2 hrs.	1.0 N/10 sodium hydroxid
5	5 c.c.	0.4 c.c. N.R.S.	0.04 c.c.	2 hrs.	1.1 N/10 sodium hydroxid
6	5 c.c.	0.5 c.c. N.R.S.	0.04 c.c.		1.2 N/10 sodium hydroxid

immune serum and 0.04 of a cubic centimeter of complement were shown to be incubated with 5 cubic centimeters of a 5 per cent. suspension of the specific corpuscles, the acidity figure is 1.4. On substitution of normal serum for the immune, five times the amount (0.5 of a cubic centimeter) does not produce the above acidity, 1.2 being the maximum acidity figure. This, again, shows the special susceptibility of the hen corpuscles to the antihen serum, considered from a lipolytic point of view.

The question naturally arises as to the identity of the acids causing the acidity of the above solutions. We believed from the first that we were dealing with fatty acids, but since albuminous substances were always present as constituents of the erythrocytes, we wished to exclude the possibility of amino acids. To accomplish this, definite amounts of corpuscles, immune serum, and complement

were incubated for one hour, and one half of the resultant fluid was immediately titrated for its acidity, the other half was extracted with ether, and the acidity of the portion thus treated was tested and examined for its acid strength after separation from the ether used for extraction. The results, as recorded in table VI,

TABLE VI.

5 per cent. hen corpuscles.	Immune serum.	Guinea pig complement.	Incubation.	Acidity before extraction with ether.	Acidity after extraction with ether.
20 c.c.	5 c.c.	25 c.c.	1 hr.	1.2 per cent. N/10 sodium hydroxid.	0.3 per cent. N/10 sodium hydroxid.

show that the ether took up the bulk of the acidity. Consequently it was definitely established that we were dealing with fatty acids almost exclusively.

The appearance of fatty acids in a suspension of red blood cells after incubation with blood serum is probably due to the lipase of the serum acting upon the lipoid constituents of the erythrocytes. The fact that this acid production is much greater when immune serum and the specific corpuscles are used is explainable only by the conception that by immunizing animals to foreign red blood cells there is not merely an increase in the non-specific lipase content of the serum of the treated animals, as reported by others, but that besides there is formed in reaction to the lipoids or lipoid combinations peculiar to the cells used, a lipase which acts specifically upon these substances.

CONCLUSIONS.

1. Erythrocytes have definite lipoidal constituents and lipoidal combinations peculiar to the species.

2. Certain lipoids and lipoidal combinations act as specific antigens.

3. The increase in serum lipase which occurs upon immunization of animals to foreign red cells is, at least in part, specific.

4. This specific lipase plays, no doubt, an important part in specific serum hemolyses.

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CONDITIONS GOVERNING THE GROWTH OF DISPLACED NORMAL TISSUE.*

By C. V. CRASTER, M.D.

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It is well known that normal tissue transplanted or displaced into an unusual environment may persist for a time but usually tends to disappear, while, as in the case of a skin graft upon an ulcerated surface, the tissue transplanted into normal environment often survives and carries on its functions. The question as to what factors tend to prevent the persistence and growth of displaced normal tissue seems almost as important with regard to the abnormal growth of tumor tissue as that other much discussed question as to why transplanted tumor tissues grow.

Several possibilities suggest themselves and there may well be others. First there is the question of the technique employed in the transplantation; either the circulation may be reëstablished by anastomoses of the vessels or the tissue may be merely implanted and left to care for itself, sometimes standing little chance of receiving a new blood supply until nearly all, or all of the cells have died. Naturally in the second case the chance for the survival of the tissue will be prejudiced by the lack of circulation. Nevertheless, it is under just such circumstances that tumor implantations succeed, though, as in the transplantation of normal tissues, it is nearly always after the sacrifice of most of the cells.

We recognize fundamental differences in the power of tumor cells and normal cells to grow in new situations in the body, but these are evidently not dependent upon the disturbances of circulation to which these cells are exposed in transplantation.

Two other possible influences suggest themselves through the effects of which we must still recognize a difference between the

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behavior of normal and tumor cells. It is clear that in the new site physical conditions unsuited to the life of the transplanted cells may exist. Thus to state an extreme case, if we evert the mucosa of the intestine so that it occupies the place of part of the skin and expose it to the same conditons as the skin, it tends to dry up, leaving a granulating surface which may later be covered by skin. Is it not possible that the physical conditions of moisture, etc., into which we plunge a piece of skin if we bury it in the tissues, may be just as injurious to it? For example, it seems to be a far slighter change of environment for a piece of thyroid tissue when we implant it in the abdominal muscles.

The second type of influence is that which is exerted by the fluids of the body upon the transplanted tissue in virtue of their specific characters, which apparently become increasingly hostile as we consider in turn implantations into the animal itself, into another animal of the same species, and into an animal of a different or even entirely unrelated species.

The following experiments made under Dr. MacCallum's direction were meant to bear upon the relation of these several factors to the growth of displaced normal tissues. They are incomplete and their bearing on the problem is rather indirect, but since circumstances prevent the further carrying on of the work, they may be published as they are. Since it has been observed that in the transplantation of tumors from animal to animal, the ease with which growth is produced increases, at least for a time, it was proposed to determine whether it is possible to confer such ability to grow upon a piece of normal tissue by transplanting it repeatedly in the same manner from animal to animal. Experiments were made in several ways and in considerable numbers, as is shown in the accompanying table (table I).

Pieces of skin two millimeters square were implanted under the skin of the axilla in rats, and removed at intervals for transplantation in the same way into another rat. Thus in any one case a piece of skin taken from rat *A* was transplanted into the axilla of rat *B*, and then successively at regular intervals transferred to the axilla of rats *C, D, E, F,* etc., so that it was exposed successively to the influence of the bodies of many animals, sometimes eight and nine,

TABLE I.

Skin tissue.	Number of hosts.	Total length of stay in days of each piece of tissue in foreign hosts.								
		4	8	12	16	20	24	28	32	36
1	4				O					
2	3			O						
3	2		S							
4	4				D					
5	3			S						
6	3			O						
7	5						D			
8	4					O				
9	7							O		
10	5					O				
11	9								D	
12	5					O				
13	2		D							
14	4				S					
15	4				O					
16	7							D		
17	3			D						
18	5					O				
19	2		O							
20	3			S						
21	4				O					
22	2		O							
23	5					O				
24	6						O			
25	3			O						
26	3			S						
27	4				O					
28	5					O				
29	5					O				
30	6						D			
31	2		S							
32	4				O					
33	3			O						
34	5					O				
35	3			S						
36	4				O					
37	6								D	
38	3			O						
39	4				O					

Tissue was removed at intervals of four days.
D = disappeared.
O = necrotic on removal.
S = apparently healthy on removal.

just as transplanted tumor cells in their various generations pass through many animals. In another series irregular intervals of two, four, six, eight, and ten days were chosen, and sometimes as many as five hosts were passed through (table II). In most instances tiny portions of the mass were removed at each transplantation in order

TABLE II.

Skin tissue.	Number of hosts.	Total length of stay in days of tissue in foreign hosts.																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	2				S				O												
2	5			S		S			S					S			O				
3	3							S					S				O				
4	2				S				O												
5	4			S				S				S			O						
6	3				S				S				O								
7	3							S				S				O					
8	5			S		S			S									O			
9	3				S			S		O											
10	4			S				S				S				O					
Testis tissue.																					
11	3			S		S				O											
12	2			S				O													
13	3				S			S				D									
14	4			S		S				S		O									
15	1				O																
16	2				S				O												
17	3			S		S				D											
18	2				S			O													
19	2			S		O															
20	1				D																
6																					

Skin tissue was passed through animals at various periods of time.
D = disappeared.
O = necrotic on removal.
S = apparently healthy on removal.

to observe progress microscopically. In some cases the whole skin was not used but merely thin shaved skin grafts, such as surgeons use in covering ulcers.

In all cases the implanted tissue became surrounded by a capsule of connective tissue, sometimes with many wandering cells and some giant cells, and in some cases the skin was found to have rolled together so as to form a small cyst. Microscopically, steady retrogression appeared to take place in all the skin grafts, no matter how few or how many hosts the tissue had passed through. Signs of tissue change and autolysis were apparent in the loss of nuclear staining and the fusion of the cells into a homogeneous structureless mass. The necrosis took place first in the lower layers so that especially when a cyst had been formed the stratum corneum persisted longest. There was in no case any obvious evidence of proliferation

of the skin, and although in some cases the mass retained its vitality for twelve to sixteen days through the third or fourth transplantation at four day intervals, necrosis was apparent in all after the sixteenth day. Steady absorption appeared to take place in all the specimens which did not disappear more rapidly through necrosis.

These experiments tend to show that the repeated transplantation of the same piece of normal tissue confers no virulence upon it. It does not tend to grow any better in succeeding hosts, and disappears after about the same term as though it had been left in one animal.

Similar experiments were made with fragments of testis, which resulted in exactly the same way except that the testis seemed to undergo a far more rapid metamorphosis into a structureless mass (table II).

A second series of experiments was undertaken to determine the effect upon an animal of repeated implantations of skin with regard to its receptivity for the growth of such skin. In these experiments each implanted piece of skin was removed at the end of twelve days, when the fresh graft was substituted. Microscopical examination showed that necrosis began earlier in the later graft than in the earlier.

In order to estimate the part played by the physical conditions which surround the displaced skin graft, attempts were made to restore such grafts to their original relations, after they had lain for varying periods under the skin, by removing the skin which covered them. In other words, a window was cut to expose the pieces of skin which had been buried with the epidermal side out, and their corners were stitched to the corners of the window so that when the granulations had been scraped from their surfaces they lay in the normal position and environment of skin, and hence should have behaved like ordinary superficial skin grafts, differing only in that they had been buried for some time.

It was found, as is shown in table III, that the pieces of skin would heal to the underlying surface and their edges would unite with the adjacent edges of the surrounding skin if they had been buried not more than twenty-four hours. After burial for 36, 48, 60, and 72 hours, and 4, 6, 8, and 12 days, they invariably failed to survive. On being exposed, pieces of skin which had been buried

TABLE III.

Rat.	Hours after operation.					Days after operation.			
	24	36	48	60	72	4	6	8	12
1	S								
2					O				
3			O						
4	S								
5					O				
6	S								
7									
8	O								
9			O						
10				O					
11	S								
12					O				
13	O								
14									
15									
16	S								
17									
18	S								
19			O						
20									
21	O								
22	O								
23	O								
24	O								
25						O			
26							O		
27								O	
28									O

These were unsutured at the edges.

S = successful.
O = negative (sloughing).

longer than twenty-four hours were found white and opaque but not softened. They invariably dried up into a crust, even when covered by a protective dressing.

In these cases the implanted skin was not only deprived of its circulation for a time but subjected to the somewhat unfavorable action of implantation into another animal of the same species. In order, therefore, to eliminate both these factors and to retain only the change in physical conditions, an incision was made through the skin around a square of two centimeters and the neighboring skin was lifted up and sutured over it so as to cover this island of skin which now belonged to the same animal and retained its circulation practically intact (table IV).

The table shows that such buried skin retained its vitality much

longer than that in the preceding series, for on removing the covering skin so that the buried portion was exposed through the window, many survivals were secured by union with the adjacent skin. This was possible, however, only up to the twelfth day of burial; after

TABLE IV.

Rat.	Days after operation.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1		S																	
2			S																
3		O																	
4				O															
5		S																	
6												O							
7							S												
8		O																	
10												S							
11			O																
12													O						
13												S							
14										S									
15																			O
16														O					
17																	O		
[1]18												O							
[1]19											O								
21												S							
22															O				
24																O			
[1]25									O										
[1]26										O									
27							S												

[1] Sloughed through.
S = successful.
O = negative.

that the islands of buried skin were found to be incapable of resuming their function when exposed, and all of them became necrotic and dried up into crusts.

A quite similar result was obtained in another way, as is shown in table V.

Large flaps of skin, three centimeters long and one centimeter wide, with a broad pedicle were raised from the abdomen and inserted into the peritoneal cavity through a transverse incision, the pedicle being formed by the mid-thoracic portion of the skin. The flap was fixed with the epithelial surface in contact with the serous

TABLE V.

Rat.	Days after first operation when skin was replaced.																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1			S																						
2						S																			
3																									
4																				O					
5															O										
6													O												
7															S										
8									S																
9																O									
10																S									
11							S																		
12																O									
13													O												
14															S										
15																O									
16																		O							
17																									
18																									
19																			O						
20																									
21													O												
22																						O			
23																									
24																									
25																									
26																O									
27																									
28																									

Twenty-eight colored rats were used.
S = successful.
O = negative.

surface of the anterior abdominal wall and care was taken not to constrict the pedicle. At another operation the pedunculated flap was drawn out and placed in its former position on the surface after various periods, as is shown in the table. The limit of vitality in this altered position seems to be sixteen days, for many of the flaps resumed their function up to that time, but none after having been buried longer.

Injections of the blood-vessels in some of these animals, made while the flap was in its intraperitoneal position, showed the existence of a good circulation.

SUMMARY.

We are unwilling to draw any very definite conclusions from the experiments, partly because they show a survival of implanted skin of so much shorter duration than that which seems to occur in the case of spontaneous implantation cysts, and partly because the method of reëxposing the submerged grafts is rather a crude way of testing their vitality. Nevertheless, the following points seem clear:

1. The repeated transplantation of a piece of skin from one animal to another confers no exceptional power of growth upon that skin.

2. The repeated implantation of skin into one animal decreases, if anything, its receptivity for such grafts.

3. The burial of skin in the interior of the body causes, after a time, a change in the skin of such a nature that it cannot resume its normal function as an external covering tissue, even when its circulation is well maintained and it is buried in the body of the same animal. The experiments do not determine how long the cells of the skin actually remain alive, and indeed it is conceivable that the mere maceration of the protective horny layer puts the skin, when reëxposed, into the position of a moist tissue, such as the intestinal mucosa, so that it readily dries up and succumbs. Nor do the experiments throw any light upon the possible existence of cytolytic substances in the circulating fluids, although, naturally, the idea of such an action has always been present in our minds in observing the gradual loss of vitality in the transplanted tissues.

TESTING FOR EPINEPHRIN (ADRENALIN) IN BLOOD. COMPARISON OF PLASMA AND SERUM.*

By G. N. STEWART, M.D.

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Certain observers have stated that the pressor effect of defibrinated blood or serum is due to substances produced in clotting. If this be so, it is obvious that the complication introduced by this property in testing for epinephrin in blood, which was discussed in a previous paper,[1] can be avoided by preventing the blood from coagulating by the addition of some substance which does not itself interfere with the epinephrin reactions. I have put the question to the test as regards the two objects, intestine and uterus, employed in the previous work, by comparing the action of blood or plasma prevented from clotting by hirudin with the action of the same blood after defibrination or of the serum separated from it. Both dog blood and human blood were used and the result was the same in both cases; that is, that the tone-increasing power of the unclotted blood or plasma for segments of rabbit intestine and uterus was practically identical with that of the defibrinated blood or serum. Possibly in some instances the increase of tone in the intestine produced by the plasma or by the uncoagulated blood was somewhat more transient than that produced by the serum or defibrinated blood. But this is by no means constant and in any case the difference was quite inconspicuous. Serum separated spontaneously from the clot had, as was to be expected, a precisely similar action to that of serum separated by the centrifuge from the defibrinated blood.

It follows from these results that, at least so far as heterologous blood is concerned, no advantage is to be gained by using hirudin blood or plasma instead of defibrinated blood or serum in testing

* Received for publication, July 1, 1912.

[1] Stewart, G. N., *Jour. Exper. Med.*, 1912, xv, 547.

for epinephrin by the rabbit intestine and uterus reactions. A further consequence of these observations is that the substance or substances responsible for the tone-increasing power of serum on intestine and uterus segments initially beating in Ringer's solution are not produced in the process of coagulation but preëxist in the unclotted blood. In proof of these statements the protocols of two experiments, one with dog and the other with human blood, are cited, and specimens of the tracings reproduced.

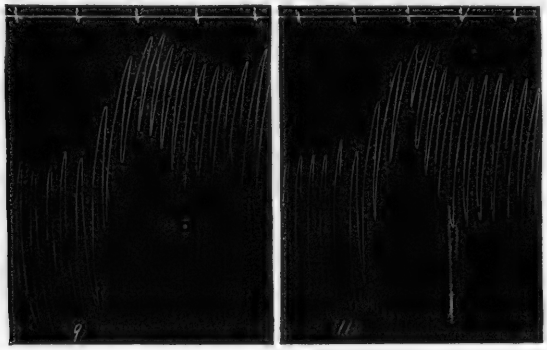
Experiment I.—A solution of hirudin in Ringer's solution containing 0.01 gm. in 1 c.c. was made. The hirudin, just obtained from the importer, was sealed in a glass tube, which was broken to make the solution.

At 11 A. M. 25 c.c. of blood from the carotid artery of a small bitch (under ether anesthesia) was run into 5 c.c. of the hirudin solution. The animal had a large goiter and the flow from the enlarged carotid was very rapid. Some blood was first allowed to escape from the cannula before collection was begun, in order to avoid all risk of the presence of clot. The blood was mixed well with the hirudin solution. No clotting occurred, even in several days. Immediately afterwards, another specimen of blood was collected and defibrinated. Ringer's solution to the amount of one fifth of the volume of the defibrinated blood was added to it so that it should be diluted to the same extent as the hirudin blood. Then the rest of the blood was at once drawn from the animal and allowed to clot after the addition of one fifth of its volume of Ringer's solution. Serum was separated immediately by the centrifuge from the defibrinated blood, and plasma from the hirudin blood. The yield was large and in both cases the liquid was free from blood pigment. The same was true of the serum from the clot, which separated very quickly.

At 2.25 P. M. tests were begun with an intestinal segment of a rabbit. Specimens of the curves are given in text-figures 1 to 3. The smaller apparatus was used, 2 c.c. of liquid being always run into it. The weight of the intestine preparation was 0.46 gm.

At observation 9 (text-figure 1) Ringer's solution in which the preparation was beating was replaced by serum from the defibrinated blood. After washing, the preparation was again beating in Ringer's solution when, at 11, this was replaced by plasma from the hirudin blood. It will be seen that the maximum increase of tone produced by the plasma was almost the same as that produced by the serum. At 13 (text-figure 2) hirudin blood replaced the Ringer and caused a considerable increase of tone. After washing, the preparation was again beating in Ringer's solution when this was replaced, at 15, by the defibrinated blood. The initial tone of the preparation before the addition of the defibrinated blood was less than its initial tone

before the addition of the hirudin blood. (The exact relations of the two curves to the base line are preserved in the figure and no change in position of the lever was made between the two observations.) It might, therefore, be expected that if the tone-increasing power of the two specimens was really equal, the absolute increase



All the tracings are to be read from left to right. Time is marked in half minutes.

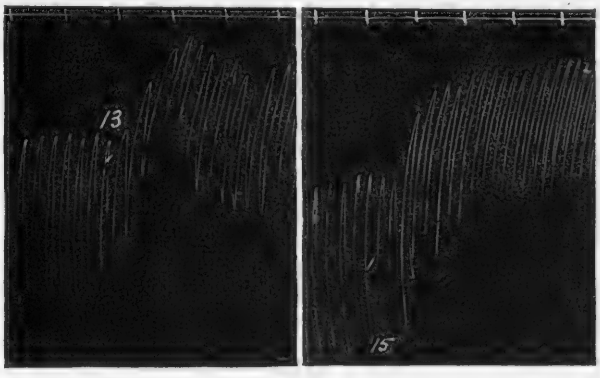
Text-figures 1 to 3 were obtained from the same rabbit intestine preparation; text-figures 4 to 6 from an intestine preparation from another rabbit. The magnification of the contractions by the lever was somewhat more than twice as great in text-figures 4 to 6 as in 1 to 3. The smaller apparatus holding 2.5 c.c. without the preparation was used throughout. 2 c.c. of the liquid to be tested, *i. e.*, enough to replace all of the liquid initially present, were always added. At the beginning of each observation the preparations were beating in Ringer's solution, the serum, etc., if previously employed, having been thoroughly washed away with Ringer.

TEXT-FIG. 1. At 9 serum from defibrinated dog blood replaced Ringer. At 11 plasma from hirudin dog blood replaced Ringer.

would be greater with the defibrinated blood, as is seen to be the case. On the other hand, the maximum height is greater for the hirudin blood. In other observations it was shown that where the initial tone of the preparation is the same, the absolute increase of tone produced by hirudin plasma is practically the same as that produced by serum from defibrinated blood.

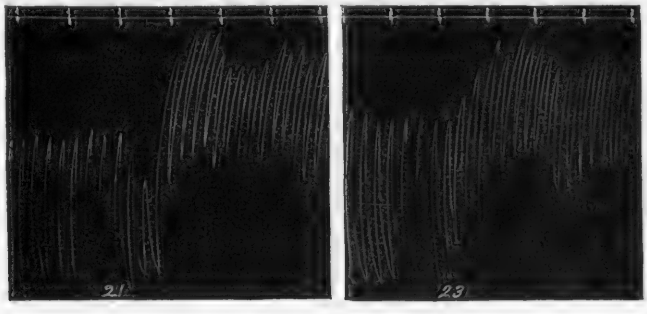
In text-figure 3 at 21 serum from the clot replaced Ringer's solution. After washing the preparation the Ringer's solution was

replaced, at 23, by hirudin plasma. The initial tone of the preparation before 23 was somewhat greater than before 21, and the same remark applies to the comparison that has been made in comparing



TEXT-FIG. 2. At 13 hirudin dog blood replaced Ringer; at 15 defibrinated dog blood replaced Ringer.

observations 13 and 15. The magnification of the contractions in figures 1 to 3 is somewhat less than half the magnification in text-



TEXT-FIG. 3. At 21 serum from clotted dog blood, at 23 plasma from hirudin dog blood replaced Ringer.

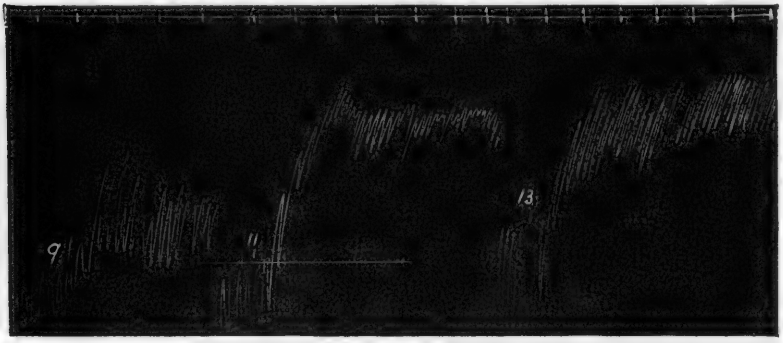
figures 4 to 6. The higher magnification was used in most of the experiments in the previous paper. This is pointed out lest the

effects in text-figures 1 to 3 might be underestimated by a reader who looks casually at the tracings.

The uterus tests showed no constant difference between the various specimens.

Experiment II.—Blood was obtained by puncture of an arm vein from F. W., a man aged 44 years, with mitral insufficiency and a history of lues several years ago.[2]

Just before the vein was tapped, 0.04 gm. of hirudin was dissolved in 4 c.c. of Ringer's solution, and 22 c.c. of blood were then run into the solution. The test-tube was agitated constantly as the blood ran in so as to insure thorough mixture of the blood and hirudin solution. The flow of blood was free. The first portion coming through the cannula was rejected. No clotting whatever



TEXT-FIG. 4. At 9 serum from F. W.'s clotted blood, at 11 his hirudin plasma, at 13 serum from his clot, replaced Ringer.

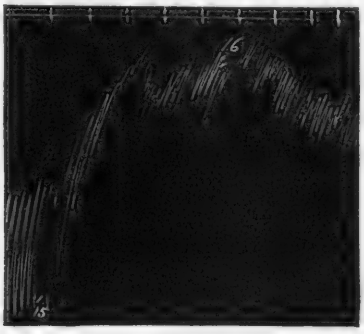
occurred, even in several days. As soon as the hirudin specimen had been obtained, 70 c.c. of blood were at once collected from the same man in another vessel, and allowed to clot. 13 c.c. of Ringer's solution were added to it. Both specimens were immediately conveyed to the laboratory and put in the ice chest over night. Abundance of hemoglobin-free serum and plasma separated.

At 11 A. M. the following morning tests were begun with an intestine preparation from a rabbit. The preparation weighed 0.44 gm. The smaller apparatus was used.

Observation 9 (text-figure 4) shows the effect of replacing Ringer's solution by serum from the clot, and 11 the effect of re-

[2] I am much indebted to Dr. Bucher, of the City Hospital, for aiding me in collecting this material. While Dr. Bucher attended to the tapping of the vein, I gave undivided attention to the proper collection of the hirudin blood.

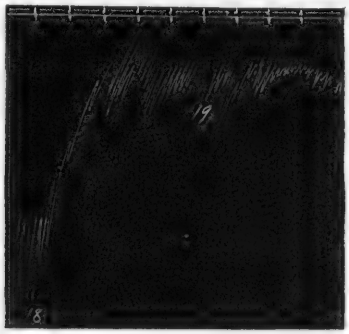
placing Ringer's solution by hirudin plasma. On the face of these two observations alone, the hirudin plasma is so far from being inferior in tone-increasing power that the increase is quite twice as great as that caused by the serum. This, however, is not a fair comparison. For 9 was the first observation in which this intestinal segment had come into contact with serum, and it is sometimes seen that the effect of the first serum bath, particularly in the case of a segment which is not beating very strongly, is to increase the power of response of the preparation to subsequent immersions in serum. This part of the tracing is reproduced here merely to illustrate the



TEXT-FIG. 5. At 15 Ringer was replaced by F. W.'s hirudin plasma, and this at 16 by serum from his clotted blood.

point that repeated observations are essential if errors of interpretation are to be avoided in this kind of work. At 13, after washing the preparation, the Ringer's solution was replaced by serum from the clot. The increase of tone is perhaps slightly greater than in observation 11, but the difference is no more than may be found in successive observations with the same serum. At 15 (text-figure 5) Ringer's solution was replaced by hirudin plasma, which at 16 was replaced by serum from the clot. In text-figure 6 this experiment was performed in the reverse order, serum from the clot replacing Ringer's solution at 18 and being itself replaced by hirudin plasma at 19. If the tone-increasing power of the serum had been conspicuously greater than that of the plasma, the rise at 16 ought to

have been decided and sustained, whereas at 19 there should have been a marked fall. On the contrary, there is a slight rise at 19 just as at 16, a rise of the same order of magnitude as is caused by simple renewal of one and the same medium, and the increase of tone after 19 is actually better sustained than after 16.

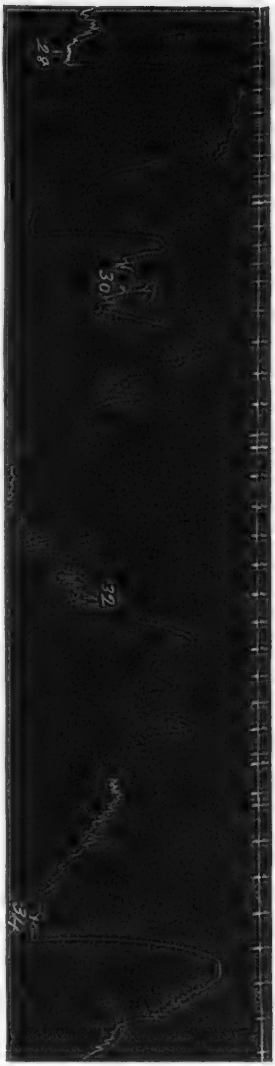


TEXT-FIG. 6. At 18 Ringer was replaced by serum from F. W.'s clotted blood, and this at 19 by his hirudin plasma.

Text-figure 7 illustrates the uterus tests with the same specimens of hirudin plasma and serum from F. W. The uterus preparation (a segment of a horn) weighed 0.58 of a gram. The rabbit was a young adult, not pregnant. The smaller apparatus was used.

At 28 hirudin plasma replaced Ringer's solution. A great increase of tone ensued. After washing, the preparation was again beating in Ringer's solution when, at 30, serum from the clot was run in. Starting from a considerably higher level, the absolute increase of tone is not so great as that produced by the plasma at 28, but is nevertheless quite distinct. At 32 hirudin plasma replaced Ringer's solution at a point corresponding to a somewhat greater tonic contraction of the segment than before observation 28. The rise, while considerable, is less than in 28. At 34, after washing, the tone of the segment was less than in any of the previous observations and the addition of serum was followed by a rise of about the same absolute amount as that caused by the plasma in observation 28.

Certainly in those observations there is nothing to support the idea that substances capable of increasing the tone of the uterus are produced in blood during coagulation. If anything, the plasma caused a greater and more persistent increase of tone than the serum. The difference, however, lay within the limits of variation in such observations even with one and the same liquid.



TEXT-FIG. 7. Uterus preparation. At 28 Ringer was replaced by F. W.'s hirudin plasma, at 30 by serum from his clot, at 32 by his hirudin plasma, at 34 by serum from his clot. After each observation the preparation was thoroughly washed with Ringer.

I have no experience of the perfusion method introduced by Läwen and developed by Trendelenberg, and I am, therefore, not in a position to discuss O'Connor's[3] statement that the pressor effect on perfused frog blood-vessels is almost entirely absent if the blood has been prevented from clotting. He says that the same is true of the tone-increasing power on the rabbit intestine and uterus. He worked, however, in the case of the intestine and uterus with homologous blood. From the point of view of this paper, only the action of heterologous serum and plasma is of interest, since in clinical testing the blood must always be heterologous to the test object. My experiments, therefore, do not strictly compare

[3] O'Connor, J. M., *Arch. f. exper. Path. u. Pharmakol.*, 1912, lxvii, 195.

with O'Connor's. It is, nevertheless, noteworthy that he makes the following statement:

"Es ist aber keineswegs leicht, in allen Fällen sicher reines Plasma zu gewinnen und die Resultate fallen nicht eindeutig aus, wenn bei der Gewinnung des Plasmas nicht jeder Zellzerfall vermieden wird; aus diesen Grunden ist es auch schwierig, die Unwirksamkeit des Hirudinplasmas auf Darm und Uterus zu erweisen; bei dem Zusammenbringen des Plasmas mit den in Ringerlösung suspendierten Organen entsteht durch die Anwesenheit der Thrombokinasen in den Geweben leicht Gerinnung. Dennoch ist es mir gelungen, sowohl am Darm als am Uterus einwandfreie Versuche zu erhalten."

I can only say that in my experiments every care was taken to collect the hirudin blood under the best conditions. The large dose of hirudin was purposely chosen to insure the absence of the slightest trace of clotting; also, as already mentioned, the flow of blood was very free. Not the slightest indication of clotting was seen at any stage of the experiments. Plasma which had once come into contact with the intestine or uterus preparation was never used a second time. The almost exact quantitative agreement in the action of the plasma and serum appears to me to be a decisive argument against the possibility that the effect of the plasma was due to a certain amount of "cell destruction" which had not been avoided. Had the plasma exerted an effect always conspicuously smaller than that of the serum such a possibility might have been entertained, but it is difficult to see how the effect could be the same unless it is independent of clotting. That occasional instances may occur in which hirudin blood or plasma apparently causes a relatively slight increase of tone while defibrinated blood or serum causes a large increase may be granted. But the converse observation may also sometimes be made, and in interpreting such results the fact that the intestine or uterus preparation varies from time to time in its power of response to one and the same liquid must not be forgotten. For instance, as already pointed out, in text-figure 4 the first two observations (9 with serum, and 11 with plasma), if standing by themselves, would indicate that the tone-increasing power of the serum was much less than that of the plasma, a conclusion which certainly would be erroneous. Suppose that one had happened at 9 to add plasma instead of serum, there is scarcely any doubt that practically the same curves as were actually written would have

been obtained. Had the experiment stopped here the interpretation might have been put upon it that hirudin plasma has a far smaller tone-increasing power than serum, and this conclusion would also have been unjustified. Observation 9, as was shown by subsequent tracings, is an anomaly in the series and must not be used in the comparison. If it were used, it would permit precisely the opposite conclusion to that which O'Connor deduced from his observations. These remarks are far from being intended as a general criticism of O'Connor's interesting paper. His conclusion that epinephrin cannot be demonstrated with certainty in the peripheral venous and arterial blood agrees entirely with that drawn in my previous papers.[4] Even if it be admitted, as O'Connor says it must be, that it is difficult to avoid errors of technique in obtaining plasma suitable for use in the intestine and uterus reactions, this alone is sufficient to show that in epinephrin-testing for clinical purposes by means of these reactions no advantage can be gained by endeavoring to substitute plasma for serum.

[4] Stewart, G. N., *Jour. Exper. Med.*, 1911, xiv, 377; 1912, xv, 547.

LIPOID METABOLISM IN THE DEVELOPING CHICK AND ITS RELATION TO CALCIFICATION.*

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PLATES 59 AND 60.

While working with a chick embryo of nineteen days' incubation, my attention was attracted by the brilliant yellow color of the liver. The color was like that of the adrenal cortex, or the corpus luteum, and this resemblance impelled me to investigate more closely the condition of the liver cells. A small fragment of the liver was teased and examined between the Nicol prisms of a polarizing microscope. The result is illustrated by figure 1. With the Nicol prisms uncrossed the field was filled with many small, rather refractile droplets (figure 1, *A*), and these upon crossing the prisms exhibited with great clearness and beauty the phenomenon of double refractivity (figure 1, *B*). Sudan III was now added to the preparation and the droplets gradually stained a vivid yellowish red. It was clear, then, that the liver of the chick embryo of nineteen days incubation contained an abundance of fatty matter of an anisotropic lipoidal nature. This discovery was quite surprising, for as far as I am aware, the occurrence of anisotropic lipoids in the parenchyma cells of the liver has never been described.

Much has been written within recent years concerning the physical and chemical properties of the so called lipoids, and a great deal has been learned in regard to their physiological and pathological occurrences in the body. I need not review even the principal contributions to the subject at this time, for the writings of Adami (1), Lorrain Smith (2), and others in English, and the contributions of numerous German authors, Kaiserling and Orgler (3), Aschoff (4), Bang (5), Kawamura (6), and still others are very

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recent and readily accessible. I have summarized in another place (7) the results of this work.

Surprisingly little of a precise and definite nature has been learned concerning the rôle of the lipoids in metabolism. The human adrenal cortex has long been known to contain lipoidal substances in abundance, but although their occurrence is now recognized as normal and not due to "fatty degeneration," no satisfactory explanation of their function has been advanced. Lipoids are present, in interesting qualitative and quantitative variations, in the adrenal cortex of most, if not all animals and there can be no doubt that they subserve a definite metabolic function. They are likewise constantly found in the corpus luteum cells, in the interstitial cells of the testes, in the thymus during regression, and frequently in the hypophysis. The abundance of lipoids in the ovary during pregnancy, a condition most strikingly exhibited for example by the pregnant rabbit, suggests some interrelation between the adrenal cortex and the ovary, possibly of a compensatory functional nature.

The occurrence in such abundance of anisotropic lipoids in the developing chick liver indicated a participation in some way of these substances in the metabolism of the embryo chick, and with the hope that the solving of this problem would perhaps throw some light upon the question of lipoidal metabolism in general, I undertook an investigation of the fats of the embryo chick liver from the earliest days of incubation to the time of hatching. The period of incubation of the chick is twenty-one days, and several livers from the sixth day on have been studied.

PHYSICAL AND CHEMICAL PROPERTIES OF THE FATS OF THE DEVELOPING CHICK LIVER.

As the liver of the chick embryo increases in size, it gradually assumes more and more the color of the yolk. Material derived from the yolk is gradually stored within the liver, and this material is largely of a fatty nature. The liver cells of the six day chick embryo contain numerous small fatty globules, which under the polarizing microscope are isotropic. This condition persists, the fat globules gradually increasing in size and number, until about

the fourteenth day of incubation, when many of the fat globules begin to show the phenomenon of double refractivity, or anisotropism. At first not all the droplets within the liver cells exhibit the dark maltese cross and four bright sectors which characterize the state of anisotropism; some droplets remain isotropic, while others show but the faintest indication of anisotropism. During the fifteenth and sixteenth days, however, all the numerous fat droplets within the liver become doubly refractive, until on the seventeenth day the brilliant picture illustrated by figure 1 is obtained. A section of the liver of a chick at the time of hatching (figure 2) shows the presence of many vacuoles, due to fat droplets, in the cells of the liver.

It is obvious, then, that the fat present in the chick liver during the first two weeks of incubation gradually undergoes changes in physical properties during the third week.

Similar changes in the physical characters of fatty globules have not been observed before, and it will therefore be important to follow the chemical changes which accompany this alteration of the physical state of the fat droplets. The several additions of the past few years to the microchemical methods for the differentiation of fatty substances are of great aid in such a study. They permit of a fairly accurate and rapid orientation, and while they in no wise replace chemical analyses, the latter are with difficulty applicable to most pathological studies. I have chosen, therefore, to apply at first to the study of the fats of the chick liver microchemical methods which are easily available. To this end small pieces of liver were teased into thin layers upon cover glasses and treated by the following methods: (1) Sudan III (saturated solution in 80 per cent. alcohol); (2) Nile blue sulphate (saturated aqueous solution); (3) neutral red (strong aqueous solution); (4) Smith's stain (8) as modified by Dietrich (9);[1] (5) Ciaccio's stain;[2] (6) Fischler's

[1] Two days in a saturated aqueous solution of potassium bichromate at 37° C.; wash thoroughly in frequent changes of distilled water for twenty-four hours; stain for twelve hours in Kulschitsky's hematoxylin; wash and differentiate for twelve hours in Weigert's borax-potassium-ferricyanide solution; wash thoroughly and mount in levulose.

[2] Fix for two days in Ciaccio's mixture; seven days in potassium bichromate 3 per cent.; wash thoroughly for twenty-four hours; twenty-four hours in 95 per cent. alcohol; two hours in absolute alcohol; one hour in xylol; pass back through successive alcohols to Sudan III for one hour; mount in levulose.

method for the demonstration of fatty acids and soaps. Both the original method and Klotz's modification have been used. In addition, the polarizing microscope has been constantly employed, and the chemical tests of Salkowski and Golodetz for cholesterol have been used.

Kawamura (6) has recently applied these methods to the comparative study of various types of fats, and I refer the reader to his monograph for the many details. His results, while continuing and elaborating the work of others, have shown that by the methods described, neutral fats, cholesterol esters, and phosphorized fats may be differentiated with remarkable accuracy.

The results of the study of the livers of fifty chick embryos by the methods just described may be stated briefly in tabular form.

It is clear from these reactions that the fat present in the chick liver up to the fourteenth day of incubation is not pure neutral fat, nor does it contain fatty acids or soaps in demonstrable amounts. Stained by Smith's method, which is essentially an application of Weigert's myelin-sheath stain, the fat droplets are colored bluish black with hematoxylin. Neutral fats and pure cholesterol esters do not stain by Smith's method (Smith, Kawamura, Aschoff), whereas phosphorized fats and various mixtures of these with other lipoids are readily stained by this procedure. The positive staining of the fat droplets in the liver before the fourteenth day, when treated by Ciaccio's method, is further indication of the phosphatid nature of the fat. Neutral fats and cholesterol esters react negatively to this stain.

I have concluded, therefore, that the fat in the chick liver during the first two weeks of incubation represents a mixture of lipoids in which phosphorized fats predominate. This conclusion is strongly supported by conditions in the yolk membrane to be described presently, and by certain chemical analyses which will be detailed later. From the fourteenth day on, the fat droplets in the liver gradually change in their microchemical reactions, and at the same time become anisotropic. The droplets in livers studied on successive days of incubation gradually cease to stain by Smith's method. On the twenty-first day they stain faintly gray, whereas five to six days after hatching the fat present in the liver does not stain at all by Smith's

	Nicol prisms.	Sudan III.	Nile blue sulphate.	Smith's stain.	Ciaccio's stain.	Fischler's stain.	Neutral red.	Salkowski's test for cholesterol.
Fat in the liver from the 6th to the 14th day of incubation.	Isotropic	Yellowish red	Faintly blue	Dark bluish-black	Positive	Negative	Negative	Faintly positive
Fat in the liver from the 14th to the 21st day of incubation.	Anisotropic	Yellowish red	Uncolored or faintly pink.	Faint bluish-gray to unstained	Negative	Negative	Negative	Strongly positive

method. Ciaccio's stain is likewise negative during the last week of incubation and after hatching. These droplets then correspond in their reactions very exactly to the properties characteristic of the esters of cholesterol (Kawamura, Aschoff).

They exhibit, furthermore, other characteristics of cholesterol esters. If they are warmed to about 40° C. they become isotropic, and this represents their constant clearing point. Upon cooling they again assume the fluid crystalline, anisotropic phase. Cholesterol esters, or mixtures in which these predominate, differ from other anisotropic lipoids in the ease with which they are converted by gentle warming from the anisotropic to the isotropic condition. They are heat labile, whereas other anisotropic lipoids, as we shall presently see upon considering the yolk membrane, are heat stabile.

The esters of cholesterol, as is well known, are much more resistant to autolysis than are other fats, and the anisotropic fat droplets so abundantly present in the chick liver are found to be extremely resistant to autolytic changes. They retain their anisotropic form for about ten days when autolyzed at 37° C., and then gradually change to solid crystals which are long and needle-like with blunt ends. On heating these crystals to about 50° C. they melt, and on cooling, many assume again the fluid crystalline, anisotropic phase. Some splitting of the fatty substance does occur, however, during autolysis, with the formation of pearly white, rhomboid, notched, plate-like crystals. These give the sulphuric acid and Golodetz reactions for cholesterol. When fixed in formol many of the anisotropic droplets assume a solid crystalline form.

From the evidence that has been brought forward I think it justifiable to conclude that the transformation in the character of the fat droplets of the chick liver, which begins about the fourteenth day of incubation, represents a change from a lipoidal mixture, composed largely of phosphatids, to almost pure esters of cholesterol.

RELATION OF THE CHANGES DESCRIBED IN THE FATS OF THE LIVER TO OTHER METABOLIC PROCESSES.

Questions arise as to the significance of this remarkable alteration in the character of the fat of the chick liver, and as to the meta-

bolic process with which it is associated. I regard the change as intimately associated with the process of calcification. There is chemical evidence in existence which strongly supports this view, and the impartial origin and careful nature of this evidence increases its value.

The composition of egg-yolk is as follows:

	Per cent.
Water	47.19–51.49
Solids	42.81–48.51
Fats (olein, palmitin, stearin)	21.30–22.84
Vitellin and other albumins	15.63–15.76
Lecithin (phosphorized fat)	8.43–10.72
Cholesterin	0.44–1.75
Mineral salts	0.36–3.33

Plimmer and Scott (10) have studied the transformations in the phosphorus compounds in the hen's egg during development. They find only a trace of inorganic phosphates present in the unincubated egg, whereas 60 per cent. of the phosphorus present in the hatched chick is in the form of inorganic phosphates. On the other hand, of the total phosphorus of the unincubated egg, 64 per cent. is in an organic, ether-soluble form (lecithin), whereas only 20 per cent. of the total phosphorus of the hatched chick is soluble in ether. Vitellin, a phosphorus proteid compound present in the yolk, was found to contain 27 per cent. of the phosphorus of the fresh egg, and this substance disappeared completely during incubation. It is obvious then, that the phosphorus which is used by the chick in the process of calcification must be derived from organic phosphorus compounds. Plimmer and Scott found that the phosphoprotein, vitellin, was removed from the yolk in large part during the first two weeks of incubation, whereas the phosphorized fats of the yolk disappeared rapidly only after the thirteenth day of incubation. They concluded that the glycerophosphoric acid present in the yolk in combination with fat gives rise to inorganic phosphate in the developing chicken, and is not transformed into any combination with protein.

The transformation of the phosphorus of the hen's egg from an organic into an inorganic state takes place, as shown by Plimmer

and Scott, between the thirteenth and the twenty-first days of incubation. This is exactly the period of calcification of the chick embryo, and it is during this same period that the striking physical and chemical changes which have been described occur in the fats of the chick liver. We are now in a position, I think, to suggest an explanation of these changes. The phosphorized fats or phosphatids of egg-yolk which, as we have seen, contain practically all the phosphoric acid used by the embryo in calcification, are present in the form of lecithin, or very similar bodies. The phosphorized fats are absorbed gradually by the yolk membrane and are carried to the liver, together with neutral fats, cholesterol, vitellin, etc. To render the phosphoric acid of the lecithin molecule available for calcification, it is split off, probably in the form of glycerophosphoric acid, and carried by the blood from the liver to the tissues which are to be calcified. Here the glyceryl radicle is replaced by calcium, with the deposition of calcium phosphate. The final deposition of inorganic phosphates and carbonates is what most writers regard as calcification. That this is an erroneous conception of the process of calcification is perfectly obvious from what has just been said. Surely the chemical changes which precede the deposition of inorganic phosphates are as essentially parts of the process of calcification as the terminal deposition itself.

The splitting off of glycerophosphoric acid from the lecithin molecule would free two fatty acid radicles, and these might be esterfied in part by the cholesterol present. This would explain the gradual appearance of cholesterol esters in the liver cells during the time that the phosphorized fat is disappearing.

I have no evidence bearing upon the fate of the cholin or other bases which form a part of phosphatic molecules.

The anisotropic lipoid droplets (cholesterol esters) gradually disappear from the liver after hatching, but even after fifteen days they are still abundant. They are not present in the liver of the adult chicken. It is worthy of note at this point that the adrenal cortex and gonads of the chick contain anisotropic lipoids in abundance.

YOLK MEMBRANE DURING INCUBATION.

The yolk membrane of the developing chick is roughly analogous to the fetal placenta. It surrounds the yolk when fully developed and the many highly colored vascular folds and processes into which its inner surface is thrown extend downward into the semifluid yolk. The cells forming the lining of the yolk membrane (figure 3), as seen in paraffin sections, are columnar, with the nucleus at the base, and the cytoplasm is scant and foamy. This vacuolated condition of the cytoplasm is due to the dissolving out of fat droplets. If a small piece of yolk membrane be examined with the polarizing microscope previous to the time of hatching, the large lining cells are seen to be loaded with droplets of various sizes, many of which are anisotropic. These anisotropic globules have characteristics which sharply distinguish them, however, from those which appear during the last week of incubation in the liver. The anisotropic globules in the cells of the yolk membrane do not lose their anisotropism when heated even as high as to 90° C., whereas the globules in the liver become isotropic above 40° C. They stain with neutral red, and many of them form irregular myelin figures upon the addition of water. Nile blue sulphate stains them blue. In short, the fat globules in the cells of the yolk membrane react in every way like phosphatids, and not at all like cholesterol esters.

At the time of hatching, the yolk-sac is within the abdominal cavity of the chick. An investigation made at this time of the cells lining the yolk membrane shows that they still contain abundant fat globules but these no longer exhibit the reactions of phosphatids, but now react like cholesterol esters. They are identical in their properties with the globules so abundantly present in the liver at this time. The explanation offered of the transformations which occur in the fats in the liver applies likewise to the yolk membrane after hatching. It would seem that the extraordinary demand for phosphoric acid on the part of the chick leads to a rapid exhaustion of the phosphorized fats of the yolk, and that finally the yolk membrane, like the liver, takes part in the splitting of the phosphatids absorbed from the yolk.

CALCIFICATION IN GENERAL.

The phosphoric acid used by the chick embryo in the process of calcification is derived, as we have seen, from organic phosphorus compounds, and the evidence indicates that these compounds are phosphorized lipoids. The phosphatids of the yolk are carried to the liver, and here the phosphoric acid is split off and thus rendered available for calcification. Any explanation of the process of calcification which fails to take these facts into consideration must be regarded as inadequate.

Despite the voluminous literature upon physiological and pathological calcification, there exists at present no satisfactory explanation of the process. Wells (11) has reviewed the subject, presenting the extensive work of his own laboratory upon calcification, and analyzing the work of other investigators. Analyses of bone, and of areas of pathological calcification, show that in both instances the inorganic salts present are practically identical, qualitatively and quantitatively. From this, and from the morphological similarity of the matrices of all calcified material, Wells concludes that physiological and pathological calcification are essentially identical processes, and subject to the same explanation. He regards the process of calcification as a deposition or precipitation, according to physical chemical laws, of calcium salts derived from the blood into a properly prepared matrix, which for some unknown reason possesses a selective affinity for calcium salts. He leaves out of account all chemical reactions whatsoever, basing his explanation upon physical chemical processes.

It will be seen at once that this explanation of calcification takes into account only the terminal phase of the process. It is based on the view that the actual deposition of lime salts into a matrix constitutes the process of calcification. In the strictest sense this is perhaps correct, but the conception seems to me too narrow. In the chick, for example, it would exclude the phosphorized fats from any participation whatsoever in the process of calcification.

Our conception of the process of calcification must be broadened rather than narrowed, it seems to me, if we are to understand it correctly. It is possible that both chemical and physical chemical phenomena are factors in the process, and I shall attempt to show

that the physical chemical explanation of calcification, as conceived by Wells, is not necessarily antagonistic to the presence of a purely chemical phase in the process.

We have considered in some detail the process of physiological calcification in the chick, but before attempting any generalization from the evidence acquired from this source, it will be necessary to consider the phenomenon of pathological calcification; for, as Wells clearly points out, physiological and pathological calcification are so nearly identical in the result as to render it probable that the same basic process is accountable for both.

The intima of the aorta is the most frequent site of extensive pathological calcification in man. Atherosclerotic areas in the intima contain various forms of fat; neutral fats, phosphorized fats (lecithin), and anisotropic cholesterol esters are constantly found, and crystals of pure cholesterol are usually present. Soaps are present only in traces. Several investigators have analyzed atherosclerotic aortas for one or several substances, usually for calcium salts, but I have been unable to find a single instance of a complete analysis of both the inorganic salts and the fats. A comparison, however, of the analyses of different observers yields a fairly accurate picture, the one supplementing the other.

Baldauf (12) studied the fats and calcium salts present in a series of three normal and six atherosclerotic aortas. He says:

> "It is seen that the fatty acids and lecithin are present in greatest amount in the atheromatous aortas without gross evidence of calcification, and as the calcium content increases they rapidly diminish in amount"; and he concludes that "the remarkably high percentage of lecithin in the initial stage of calcification and the fact that with increasing calcium content there occurs a coincident diminution in the percentage of lecithin has led us to suspect that the phosphate radicle may be supplied from a decomposition of lecithin."

Unfortunately, no determinations of free cholesterol and cholesterol esters were made by Baldauf; but Windaus (13), using his very accurate digitonin method, has investigated, at the instance of Aschoff, the free and combined cholesterol present in normal and atherosclerotic aortas. He established the important fact that in atherosclerotic aortas there is a true infiltration of cholesterol and cholesterol esters.

Gazert (14) has made very extensive studies of the calcium content of the aorta in atherosclerosis, but unfortunately he simply extracted the fats with ether, and did not analyze them. This work has been confirmed by Selig (15). Gazert found that both calcium and fat increased in amount during the early stages of atherosclerosis, whereas in more advanced stages the calcium increased greatly while the fats decreased. He says:

"Das Absinken des Fettgehaltes und das Ansteigen des Kalkgehaltes in der Trockensubstanz bei dem späteren Verlaufe der Atheromatose ist so stark, dass es den Eindruck macht, als würde Fett nicht nur in verminderter Menge, oder gar nicht mehr abgelagert, sondern als verschwände sogar Fett von den am stärksten erkrankten Stellen."

In reviewing the results of the work of Baldauf, Windaus, Gazert, and Selig, several facts of importance come to light, despite the fragmentary and unsatisfactory nature of their contributions considered from the standpoint of calcification. In atherosclerosis of the aorta, neutral fats, phosphorized fats (lecithin), and free and combined cholesterol are present in markedly increased amounts. As calcification progresses these substances gradually disappear. This apparent relationship between the fats and calcium salts in atherosclerotic aortas was noted by both Gazert and Selig, but they advance no explanation. Baldauf, however, suggests that the phosphoric acid of aortic calcification is derived in part, if not wholly, from the phosphatids.

There can be no doubt that phosphorized fats may readily furnish phosphoric acid for calcification; the process of calcification in the chick embryo proves this conclusively. One possible objection to Baldauf's hypothesis arises, however; namely, whether the amount of phosphorized fat present in the atherosclerotic aorta is large enough to furnish sufficient phosphoric acid to account for the amount of calcium phosphate present in aortic calcification. This would be a difficult question to solve even if accurate and complete quantitative analysis were available. For we must remember that the fatty substances present in the atherosclerotic aorta represent in large part infiltrations from the blood into diseased areas, and are not derived in any considerable quantity from tissue degeneration. The cholesterol determinations of Windaus illus-

trate well this point. Hence it is impossible to say from an analysis of an atherosclerotic aorta whether or not the phosphorized fats are capable of furnishing sufficient phosphoric acid to account for the calcium phosphate of calcification. We can only examine one stage of a process whose several factors must be constantly changing. Granting for the moment that the phosphoric acid of calcification does come from phosphorized fats, we see at once that the splitting of such fats with the formation of calcium phosphate might continue at the same time that more phosphorized fats were infiltrating from the blood. An analysis of the embryo chick liver at any one stage would reveal only a small part of the phosphorized fat available within the yolk for calcification, and the error which is here obvious is likewise present in all probability in analyses of the atherosclerotic aorta.

There is a very striking qualitative resemblance, moreover, between the various kinds of fats present in the developing chick liver and in atherosclerosis. All the factors of the process of calcification as traced in the embryo chick are present in the atherosclerotic aorta, and since we know that phosphorized fats do furnish phosphoric acid for calcification in the one instance of physiological calcification where the entire process has been studied, I am the more inclined to believe that in pathological calcification, as represented by the calcifying aorta, the process is essentially the same as in the physiological calcification of the chick embryo. The evidence, however, is suggestive rather than conclusive, and more work will be required to test thoroughly the hypothesis.

The explanation advanced in this paper of one phase of the process of calcification does not offer a solution of the question as to why certain tissues normally calcify. The same may be said, however, of all other attempts to explain physiological calcification. In this communication I have attempted to point out that physiological calcification can not be regarded as a purely physical chemical process. As has been said before, there may be two phases in the process, one chemical, in which the elements of calcium salts are elaborated, and a second, possibly physical chemical, during which they are precipitated or deposited as salts in a matrix. Thus in physiological calcification in the chick, the liver acts in an in-

termediate metabolic capacity, whereas in pathological calcification essentially the same process may take place *in situ*.

The occurrence of anisotropic lipoids in the liver of the embryo chick is not an isolated phenomenon associated with the peculiar nutritional conditions of the egg, as might be suspected. The liver of the fetal dog and the puppy after birth contains a large quantity of anisotropic lipoid substance. I have not found such substances in the liver of the fetal pig in any of the stages of development that I have examined.

The entire subject of lipoid metabolism offers an inviting field for investigation, and here, as in so many other problems, a study of the embryological conditions promises to lead us to the truth. Many hypothetical functions have been ascribed to the lipoidal substances, but the demonstration of their close association with the process of calcification gives to them a hitherto unsuspected importance in the body's metabolism. The relationship of the adrenal cortex to calcification in the adult demands investigation, and the occurrence of lipoids in the ovary during pregnancy suggests a relationship to the fetal metabolism of lipoids.

CONCLUSIONS.

The liver of the embryo chick during the first two weeks of development contains an abundance of isotropic fatty globules which represent a mixture of lipoids in which phosphorized fats predominate.

During the third week of incubation the fatty globules in the liver change their physical and chemical characters. They become anisotropic and exhibit the reactions and properties of esters of cholesterol. The phosphorized fats gradually disappear from the liver during the third week.

The phosphoric acid utilized by the embryo chick in calcification is derived from phosphorized fats. It is suggested that the phosphorized fats are split in the liver, the glycerophosphoric acid portion being liberated for calcification, while the free fatty acids are esterfied by cholesterol.

A review of chemical analyses of aortic atherosclerosis and calcification made by others, and a comparison of the conditions in

atherosclerosis with those of the developing chick liver, suggest that pathological calcification results from a splitting *in situ* of phosphorized fats with subsequent formation of calcium salts, as suggested by Baldauf.

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EXPLANATION OF PLATES.

PLATE 59.

FIG. 1. A fragment of the liver of a nineteen day chick which has been teased in glycerin. In *A* the liquid droplets are pictured as seen under the polarizing microscope with the prisms uncrossed; in *B* a similar field is represented as seen with crossed prisms (anisotropism).

FIG. 2. Paraffin section of the liver of a twenty-one day chick embryo. The liver cells show many vacuoles due to the dissolving out of lipoid droplets. Hematoxylin and eosin.

PLATE 60.

FIG. 3. Section of the yolk membrane of a thirteen day chick embryo. The thin septa extending downward from the yolk membrane are covered with vacuolated columnar cells; numerous blood islands are seen around the blood-vessels of the septa.

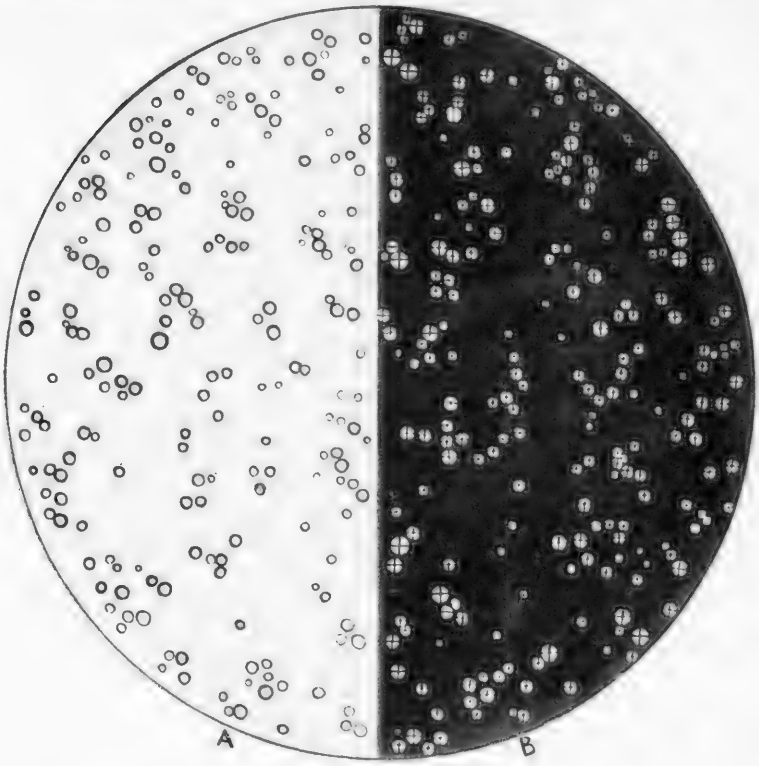


FIG. 1.

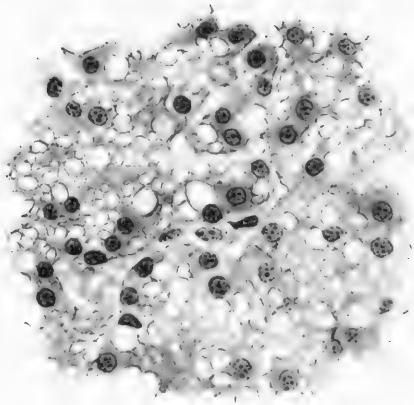


FIG. 2.

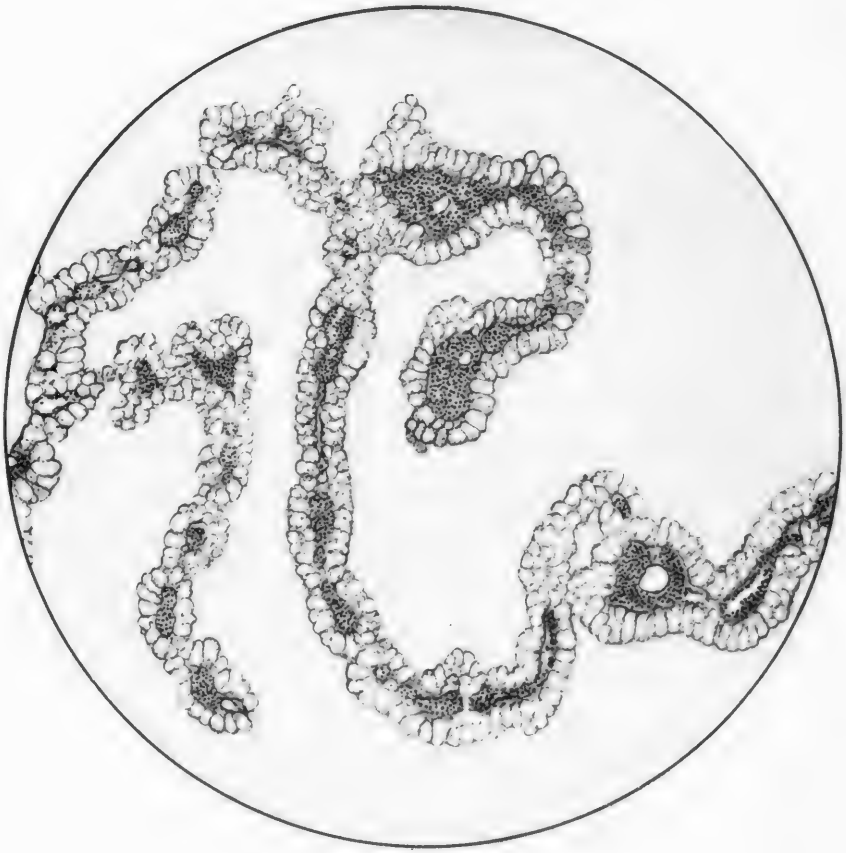


FIG. 3.

CELL PROLIFERATION AND PARASITES IN RATS.*

By F. D. BULLOCK, M.D., and G. L. ROHDENBURG, M.D.

(From the Cancer Laboratory of the Department of Zoölogy, Columbia University, New York.)

Plates 61–63.

The recent discovery of certain invertebrate parasites associated with chronic inflammatory changes at the sites of cancer predelection or in cancer itself emphasizes the relationship between chronic irritation and malignant tumor formation.

In the fourth report of the Imperial Cancer Commission, Haaland[1] describes nematode worms in the breast tissue of mice. He found these in and about areas of chronic inflammation and he gives further evidence to prove the possible effects of these organisms on tumor genesis. He sees in these irritants a possible explanation of the relatively high proportion of breast tumors in mice. Borrel[2] has also described parasites (acari) associated with early epitheliomata in man.

A large percentage of the white and hooded rats supplied to our laboratory during the past year were infected with worm parasites, of which two types have been encountered; a cestode and a nematode, the former in the liver, the latter in the bladder. Cestodes occurred in about 30 per cent. of the animals autopsied. They were encysted in various portions of the liver and were either single or multiple. The cysts were either superficially placed or embedded more or less deeply in the substance of the organ. Occasionally the cyst was found in the peritoneal cavity, below the liver, attached to that organ by a fibrous pedicle. When superficial, the cysts appeared as translucent or opaque, thin walled vesicles projecting like

* Conducted at the expense of the George Crocker Special Research Fund. Received for publication, July 2, 1912.

[1] Haaland, M., Spontaneous Tumors in Mice, *Scientific Reports of the Imperial Cancer Research Fund*, 1911, No. 4, 1.

[2] Borrel, A., *Ann. de l'Inst. Pasteur*, 1909, xxiii, 97.

blisters from the liver surface. They vary in diameter from three to ten millimeters. The contents of the cysts consist of a thick mucoid-like fluid, in which the coiled up parasite, in different stages of development, is embedded. The adult worm measures from four to five centimeters in length. Its head is triangular, and its body flattened and segmented. The cyst wall varies from a fraction of a millimeter to several millimeters in thickness.

Histological examination of the cyst wall shows a connective tissue of varying density; in some instances it is loose in structure and resembles mucoid tissue, in others it is dense and firm. The blood supply varies with the density. The connective tissue is infiltrated with numerous plasma cells and small round cells. In some cases polymorphonuclear leucocytes, particularly eosinophiles, are quite abundant. Proliferating bile ducts (figure 1) and isolated columns of liver cells (figure 2), the latter showing frequent mitotic figures, extend into the cyst wall. In old cysts the wall shows regressive changes in the form of fatty and hyaline degeneration, while calcification of the contents of the cyst is occasionally encountered.

In the region of the cyst the liver cells undergo pressure atrophy and the bile ducts show active proliferation. Throughout the entire liver there may be fatty degeneration, which is sometimes quite marked. This is the most constant pathological change and its occurrence in parts remote from the parasite indicates a toxic origin. Other changes are observed in some cases, viz., hyperemia and granular degeneration of liver cells, but these are far from constant findings. Mitoses are often quite numerous.

Degenerative changes in the kidneys, and in the thyroid and suprarenal glands have been observed. These are probably due to some other extraneous factor, as they have been found in some so called normal animals, free from liver parasites.

A recent chance finding of a parasite embryo in the pelvis of a rat kidney led us to the study of the bladder, and to the discovery of the nematode parasites. Of the thirty-six bladders examined thirty-two, or approximately 90 per cent., showed these thread worms in all stages of development. Two of the corresponding kidneys showed parasites or embryos in the pelvis. The parasites are in the bladder, either alone or associated with firm plugs of organic material, which often project into the urethra; on these plugs calcareous matter is sometimes deposited, forming irregularly shaped calculi. Upon teasing out the mucous membrane of the bladder, the living worms and embryos are isolated. The adult parasites are white thread-like organisms, tapering from head to tail, and measuring from twelve to fourteen millimeters in length. They

are sometimes found with their heads embedded in the mucous membrane of the viscus and their tails firmly fixed in the organic mass, and when freed they move actively. The urine may show desquamated cells and products of parasites, or may be negative.

Aside from the changes in the immediate neighborhood of the parasite a large proportion of the bladders showed only desquamation of the superficial cells of the epithelial layer. A few showed either distension with atrophy of the coats, due to obstruction, or hypertrophy. In several there were fatty and hyaline changes in the mucosa and submucosa. Two showed chronic inflammatory changes characterized by eosinophile infiltration. Beginning keratinization was observed in the epithelial cells of one organ. In two bladders the mucous membrane was perceptibly thickened; one showed active proliferation of the epithelial cells as indicated by numerous mitoses, but no evidence of metaplasia.

The embryo parasites are embedded more or less deeply in the epithelial layer of the mucosa and are covered by one to several layers of markedly flattened epithelial cells (figure 3). On each side of them there is a slight heaping up of cells (figure 4). The main change, however, is one of pressure atrophy of the surrounding cells, produced by the growing organism.

The only finding of special interest was made in the bladder of a rat in which we were studying the regenerative powers of the pancreas, after an experiment in which a portion of the splenic end of the pancreas was ligated and replaced in the abdominal cavity. The animal was killed twenty days after operation. Upon opening the abdomen a large encapsulated mass was found which enclosed the ligated end of the pancreas and a considerable portion of the spleen. The organs enclosed had undergone more or less complete autolysis. The viscera of this animal showed marked degenerative changes, probably of bacterial origin, since bacteria were found on microscopical examination. There was no cestode infection of the liver.

The histological examination of the bladder revealed a general hyperplastic condition of the epithelial lining and here and there small polypoid excrescences. In these regions the cells were markedly increased in number and they were all in a very active state of growth, numerous mitotic figures being encountered. The cells were heaped up one upon the other in a disorderly fashion. The lower

cells apparently invaded the pedicle of the polyp (figure 5), while here and there throughout the bladder generally the sharp line of demarcation between the epithelium and the stratum proprium was lost, giving to the whole a ragged appearance. Embryos of the parasite were found in the same area (figure 6). There was no invasion of the muscular coat. The cells were polyhedral, oval, or fusiform in shape, and desquamation of the superficial cells was marked. Such a condition of the bladder is generally considered a benign hyperplasia, but in our opinion it stands on the border line between inflammatory hyperplasia and cancer.

In man, a somewhat similar condition produced by the Bilharzia hematobia is known, though here the causative agents are the sharp pointed ova which are embedded principally in the submucosa. They produce polypoid growths which sometimes become cancerous.

What factor or factors are responsible for the marked proliferation of the cells? Mechanical irritation alone does not seem a plausible explanation. Coccidia may abound in the intestinal epithelium of rabbits without stimulating the cells to divide. If mechanical irritation alone were the factor we should expect to find the most marked changes in those bladders in which there were numerous parasites and calculi. In the animal in which this stimulation of cell growth was most marked, the irritation was slight as compared with the other bladders, for there were but few parasites and no calculus. As an explanation we may mention the fact that the focus of chronic irritation, which was parasitic in origin, occurred in a body saturated with the products of nucleoproteid autolysis.

In the case of the cestodes the absence of marked new growth of liver cells about the parasite is perhaps to be explained from the fact that in the liver the parasite provoked a marked reaction on the part of the connective tissue, the encapsulation effectually preventing further local action.

The occurrence of parasitic infections in rats and mice, coupled with the fact that they have the power of inducing inflammatory changes tending to cancer formation, may prove to be the explanation of the so called cage infections which have been recorded in various laboratories. We would in any case urge a more detailed study of the bladder in laboratory animals, believing that many small and early malignant tumors will be found.

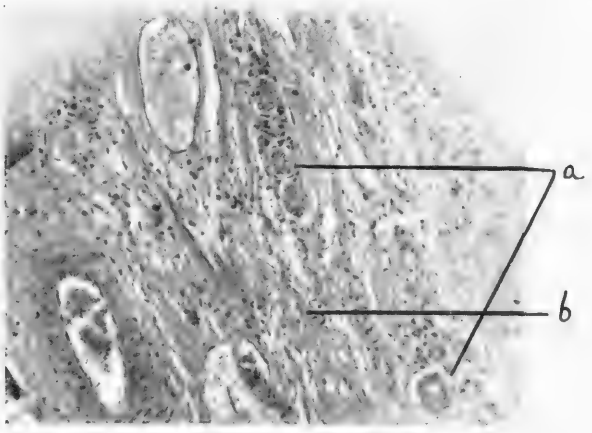
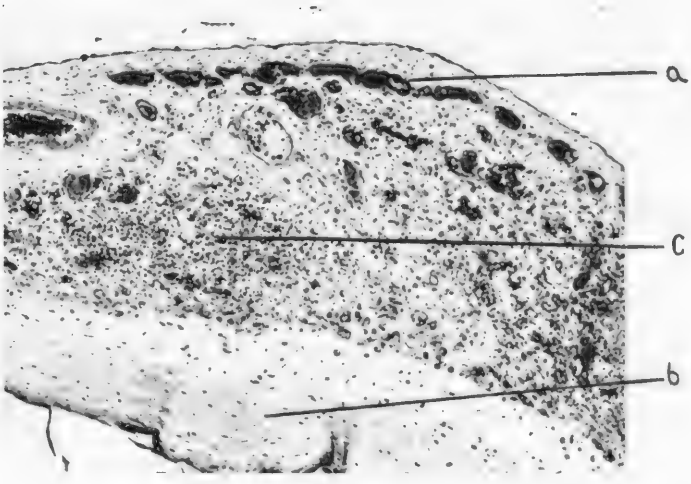


FIG. 1.



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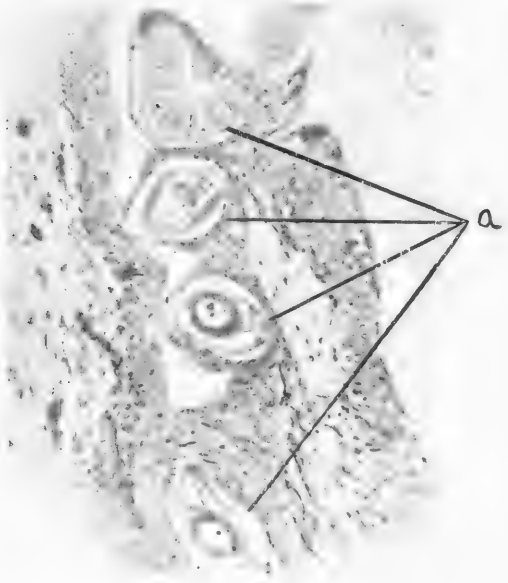
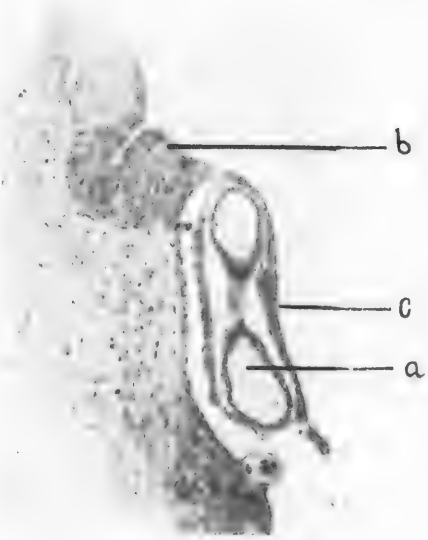


FIG. 3.



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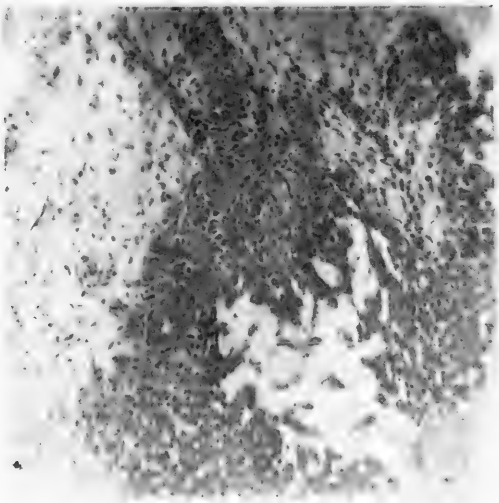


FIG. 5.

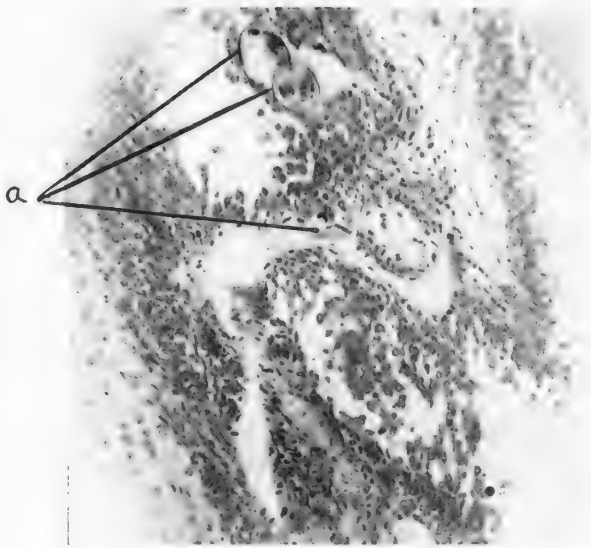


FIG. 6.

EXPLANATION OF PLATES.

PLATE 61.

FIG. 1. Section of the wall of a liver cyst showing proliferation of bile ducts (*a*); connective tissue (*b*), structure loose.

FIG. 2. Section of the wall of a liver cyst showing proliferation of liver cells (*a*); connective tissue (*b*) dense and firm; moderate round cell (*c*) infiltration.

PLATE 62.

FIG. 3. Section of the bladder wall showing the parasite (*a*) embedded in the epithelium.

FIG. 4. Section of the bladder wall showing the parasite (*a*) embedded in the epithelium and heaping up of cells (*b*) about the same. Note the thin layer of flattened epithelium (*c*) covering the parasite.

PLATE 63.

FIG. 5. Another field of the same bladder shown in figure 6, showing the invasive character of the growth about the base of the polypoid excrescences.

FIG. 6. Section of the bladder wall showing the marked proliferation of cells occurring in one instance. Note the parasite embryos (*a*) in the field.

OBSERVATIONS WITH REGARD TO THE ACTION OF EPINEPHRIN ON THE CORONARY ARTERY.*

By EDWARDS A. PARK, M.D.

(From the Department of the Practice of Medicine, College of Physicians and Surgeons, Columbia University, New York.)

PLATE 64.

The observations here reported were made partly in the course of twenty-five successful experiments concerning the vasoconstricting substances of blood serum (Janeway and Park (1)), partly in the course of a smaller number of experiments on the excised coronary artery performed directly in preparation for this work. The method employed has been the excised ring method of Meyer (2).

Previous investigators of the reaction of the coronary artery to epinephrin have used both perfusion and the excised strip or ring method of Meyer. Using the former, Schäfer (3) obtained no effect capable of definite interpretation. von Langendorff (4), despairing of his ability to overcome the great obstacle to the perfusion method, *i. e.*, excitation of cardiac contraction, abandoned that method and first applied the excised ring method to the study of the effect of epinephrin on the coronary. Wiggers (5) avoided excitation of the cardiac muscle by the perhaps questionable expedient of using an unoxygenated sodium chlorid solution as a perfusion medium; he obtained constriction. By the perfusion method Campbell (6) had at times no effect, at other times slight constriction. Brodie and Cullis (7) reported constriction of the coronary when they perfused with a dilution of epinephrin so small as not to augment cardiac contraction, but relaxation of the coronary when the strength of epinephrin was increased beyond this point. On the other hand, by the use of the excised ring method, von Langendorff (8), Eppinger and Hess (9), Pal, de Bonis and Susanna (10), Cow (11), and Campbell (12), have all

* These observations were conducted under the Maria McLean Proudfit Fellowship in Medicine. Received for publication, July 3, 1912.

demonstrated that epinephrin relaxes the excised coronary ring. While, then, the investigators by the perfusion method have obtained conflicting results with epinephrin, those using the excised ring method have obtained a uniform effect, relaxation of the coronary. Recently, however, Barbour (13) has introduced disagreement even here. Though, like his predecessors, he obtained relaxation from epinephrin when he used the coronary of animals,—ox, sheep, and pig,—he found that epinephrin used upon the coronary artery of man has a constricting effect.

Barbour's (14) investigations into the reaction of the human coronary to epinephrin require especial mention. He performed nine successful experiments on the excised rings of human coronaries obtained from three subjects in the first few hours, in one case seven hours, after death. These nine experiments he regarded as being free from error. In six of the nine he obtained constriction of the excised ring, in three no effect at all. The number of his experiments is then rather small, and only two thirds of them were of a positive nature. Yet on the basis of these experiments he assumes the general conclusion that epinephrin constricts the human coronary artery.

It would be remarkable if epinephrin produced on the coronary artery of animals one effect and the opposite effect on the coronary of man. The volume of proof required to establish so notable an exception to what has appeared to be a general law—so far as the excised ring method is concerned—must necessarily be great. The human coronaries investigated by Barbour had lain in the body for from two to seven hours after death. Who knows how the coronary arteries of animals allowed to lie *in situ* a like length of time might react to epinephrin? This point is still to be determined. The human coronaries of Barbour's experiments were pathological; all three showed intimal proliferation and one of them, in addition, calcification. Dale (15) has demonstrated that ergotoxin may alter the action of epinephrin on peripheral vessels. Is it not possible that the receptive mechanism in these pathological vessels has in some way become deranged by the disease process, and the character of the response to epinephrin has altered in consequence? But most important of all, how does the normal

human coronary react to epinephrin? Surely not until this last question has been answered can the conclusion be accepted that epinephrin constricts the coronary artery of man.

METHOD EMPLOYED IN THESE EXPERIMENTS.

The method used in my experiments does not differ materially from the original method of Meyer. Rings of the coronary artery of the ox, about three to four millimeters thick, were used instead of strips. Usually two of these were fastened together tandem fashion and suspended in a glass chamber. The lower ring was fastened to the floor of the chamber, the upper ring was connected to the short arm of a lever. The long arm of this lever recorded on a drum. The rings were bathed in Locke's fluid, which was kept constantly oxygenated and at a temperature of about 37° C. Epinephrin was introduced into the glass chamber containing the Locke's solution, either directly from a pipette or by a system of syphonage which involved withdrawal of the Locke's fluid and substitution of the desired dilution of epinephrin in Locke's fluid. The initial tonus was overcome by the use of weights. For his carotid strips Meyer used a stretching weight of eighty-six grams, which at the end of about twenty minutes was reduced to a lifting or permanent weight of about fifty grams. The best stretching weight for the ox coronary ring of about three millimeters diameter and three to four millimeters thickness is thirty grams, which is reduced at the end of fifteen minutes to a lifting load of about twenty grams.

Barbour employed a still different method to overcome the initial tonus of his coronary rings. He used a small weight, but depended chiefly on the sudden relaxation which occurs in the coronary ring when the temperature is elevated to about 42° C. In the experiments performed by the writer, the greatest pains were taken to prevent any such variation of temperature as Barbour intentionally employed.

It is worthy of mention that the coronary artery as it is found in the slaughtered animal always presents a wide lumen, in contrast to the peripheral artery which is regularly in a constricted state but with the cut ends flaring out in bell-shaped fashion. These facts

would seem to indicate an essential difference in the tonus character of the two kinds of vessel. Attention should also be directed to the difference in behavior of the two during the course of any experiment. The coronary artery is much less subject than the peripheral to unaccountable fluctuations in tone.

EXPERIMENTS PERFORMED.

As already stated, Brodie and Cullis (16) found in their perfusion experiments that epinephrin in dilutions too small to augment heart action nevertheless constricted the coronary artery. By experiments made on six ox coronaries, I have attempted to determine whether very dilute solutions of epinephrin may produce a primary constriction, but have invariably found that the primary effect of threshold values of epinephrin is relaxation.

Rings of the coronary were subjected to a series of solutions of epinephrin, beginning with 1 to 200,000,000 and increasing in strength. In every experiment the first distinguishable effect of epinephrin on the coronary was relaxation at 1 to 50,000,000, appearing as a barely perceptible temporary depression of the tracing line (plate 64, figure 1). Perhaps it would have been possible to demonstrate an effect from a solution even more dilute, by increasing the sensitiveness of the coronary ring as described later. Meyer's threshold values for epinephrin on the ox carotid were in the neighborhood of 1 to 90,000,000.

Though there is no need of further proof that epinephrin relaxes the coronary artery of the ox, certain observations in regard to the nature of this relaxation are of interest. By the simple procedure of varying the lifting load, the quantitative effect of epinephrin on the coronary artery can be made to vary within wide limits. When, for example, the stretching load of thirty grams was reduced to a lifting load of twenty-nine grams (instead of the usual lifting load of twenty grams), it was found that epinephrin produced a relatively small relaxation, for the obvious reason that the coronary rings were already in a much relaxed condition on account of the heavy lifting load. But when, on the other hand, the stretching load of thirty grams was reduced to a lifting load of only two grams, the relaxing effect of epinephrin was again small, although the rings

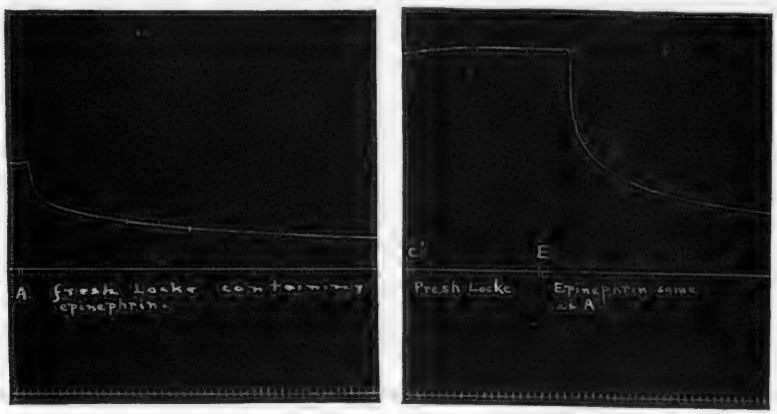
after their release from fourteen fifteenths of the original load had undergone marked shortening and were in a state of tonus ideal for the action of epinephrin. In the latter instance the rings failed to react maximally, not because they had been insufficiently affected by the epinephrin, but because a weight sufficient to produce elongation was lacking.

In a strict sense, then, the action of epinephrin on the ox coronary cannot be regarded as being dilatory, as no spontaneous increase in the diameter of the vessel occurs under its influence. The action seems analogous to the effect of epinephrin upon the intestinal musculature, and seems to be inhibitory. It cannot be thought of, however, as producing a complete abolition of tone, for if tone were completely abolished by epinephrin, as great a degree of relaxation would be produced by a light as by a heavy weight. The action on the coronary, therefore, seems to be in the nature of a sudden lowering of tone, so that the musculature offers but feeble resistance to any relaxing force. Epinephrin produces a very considerable temporary weakening of the arterial muscle, but does not completely paralyze it.

In this connection the work of Kepinow (17) is of interest. His experiments indicated that the combined action of epinephrin on the receptive substance and hypophysis extract on the contractile substance produced an effect on the peripheral vessel greater than the sum of their single effects. On the other hand, there is no doubt that epinephrin seriously impairs the effect of certain substances, which constrict the coronary by direct action on its smooth muscle. Epinephrin in sufficient dosage may hide the constrictor effect of these stimulants of smooth muscle entirely, or modify it to a great extent.

But even when the weighting of a given ring of ox coronary is constant, I have observed that the reaction of the artery to epinephrin may vary in the course of a single experiment, without change in experimental conditions. When the weighted ox coronary rings were kept suspended in a medium of Locke's fluid for from one to two hours, a gradual constriction occurred. The causation of this constriction requires further investigation; its explanation may possibly be found in a disturbed osmotic relation.

At any rate, it is a fact that when the constriction became well developed, the effect of epinephrin was greatly intensified. When previous to the constriction a given concentration of epinephrin had produced a fall of one centimeter in the curve, after the appearance of this constriction the same concentration of epinephrin produced falls of from three to four centimeters, almost to the base level of the previous relaxation curve (text-figure 1, *A* and *B*). Such



A *B*

TEXT-FIG. 1*A*. INFLUENCE OF EXISTING TONUS OF CORONARY UPON ITS QUANTITATIVE RESPONSE TO EPINEPHRIN. *A* = strong epinephrin in Locke's solution introduced. From the point at which tracing is cut to resumption in text-figure 1*B* is an interval of three hours and ten minutes. During the first thirty minutes, dilatation remained unchanged; then gave place to a progressive, slow constriction (text-figure 1*B*.) Magnification, ×7¼ (half size reproduction).

TEXT-FIG. 1*B*. Continuation of text-figure 1*A*. Relation to base line as in text-figure 1*A*. For forty-five minutes repeated washing with Locke's fluid. *C* = final washing with Locke's fluid. *A* = reintroduction of the same solution as at *A*, text-figure 1*A*.

variations in the degree of relaxation in the same artery from the same concentration of epinephrin, but at different times, illustrate the futility of attempting to measure the effect of epinephrin in terms of the circumference of the excised ring, or of comparing reactions in a quantitative way.

Dixon (18) has shown that apocodein inhibits the constrictor effect of epinephrin, but not the inhibitory (dilator) effect on the receptive substance. If, as Brodie and Cullis maintain, both constrictor and dilator sympathetic nerves are present in the coronary artery, any given effect of epinephrin on the coronary artery must be due to the summation of the antagonistic effects on both sets of nerve endings (receptive substances). If now the constrictor endings (the constrictor receptive substance) can be inhibited by apocodein, the relaxing effect of epinephrin should be increased; but such appears not to be the case. In four experiments performed by the writer and Dr. Charles Lieb, to clear up this point, the result was uniform. In none of these experiments was the relaxation produced by a given dosage of epinephrin more extensive after treatment of the vessel with apocodein than before such treatment

A

B

Text-Fig. 2. Failure of Apocodein to Influence the Response of the Coronary to Epinephrin. Experiment 2, series *A*, April 21, 1912. Ox coronary, four successive segments. Magnification, × 15½.

A. Segments 1 and 3. Loads, stretching, 35 gm.; lifting, 20 gm. in Locke's fluid. 1. Fresh Locke's fluid introduced. 2. Epinephrin (adrenalin chlorid) to make 1 : 5,000,000.

B. Segments 2 and 4. Loads and all conditions as in the text-figure 2*A*. 1. Locke's fluid containing apocodein 0.1 per cent. 2. Epinephrin (adrenalin chlorid) to make 1 : 5,000,000. Note quantitative dilatation in *A* and *B*.

(text-figure 2, *A* and *B*). While the number of experiments is perhaps insufficient for dogmatic statement, the results obtained would seem to be a strong argument against the assumption of Brodie and Cullis.

CONCLUSIONS.

1. Excised rings of the coronary artery of the ox properly weighted react to epinephrin by dilatation.

2. This reaction of the ox coronary is not an active dilatation, but is in the nature of a sudden lowering from a high to a relatively low degree of tonus. The tonus is not, however, entirely abolished by epinephrin.

3. The degree of relaxation produced by epinephrin is not constant. It depends on several factors other than the concentration of epinephrin used.

4. It is possible to increase the sensitiveness of the coronary artery to epinephrin.

5. There is no evidence of any primary constrictor effect on the ox coronary from epinephrin in most dilute solutions, nor can the existence in this artery of any constrictor mechanism of sympathetic origin be determined by the excised ring method.

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EXPLANATION OF PLATE 64.

FIG. 1. THRESHOLD VALUES OF EPINEPHRIN. Tracing from ox coronary artery, 2 rings, showing the effects of increasing strengths of epinephrin (adrenalin chlorid special, Parke, Davis and Co.) from 1 : 100,000,000 to 1 : 5,000,000. *L* indicates substitution of Locke's fluid. Magnification × 15½. Time in minutes.

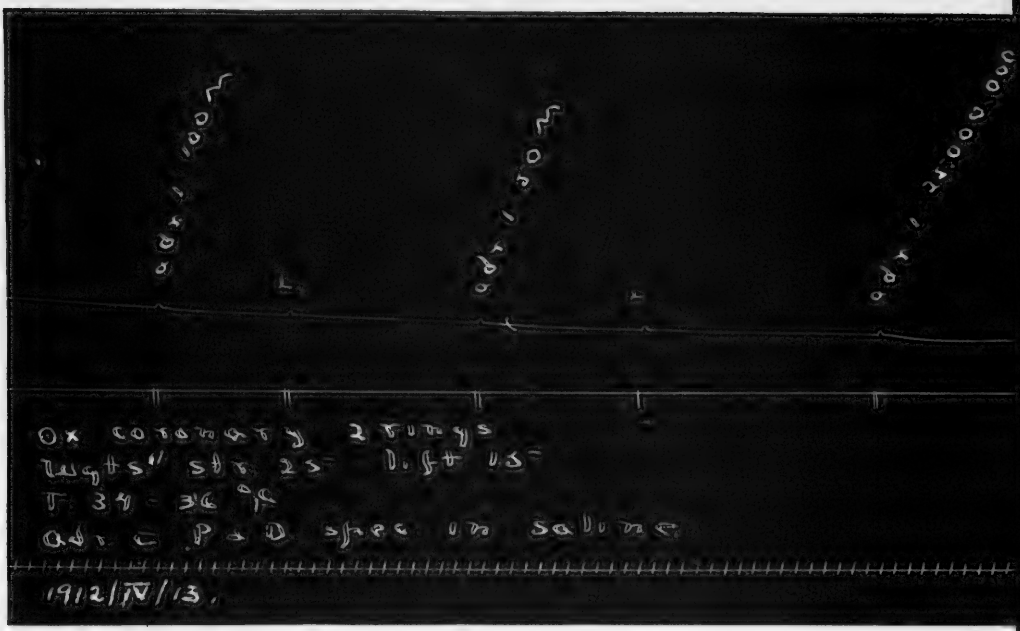


FIG. 1

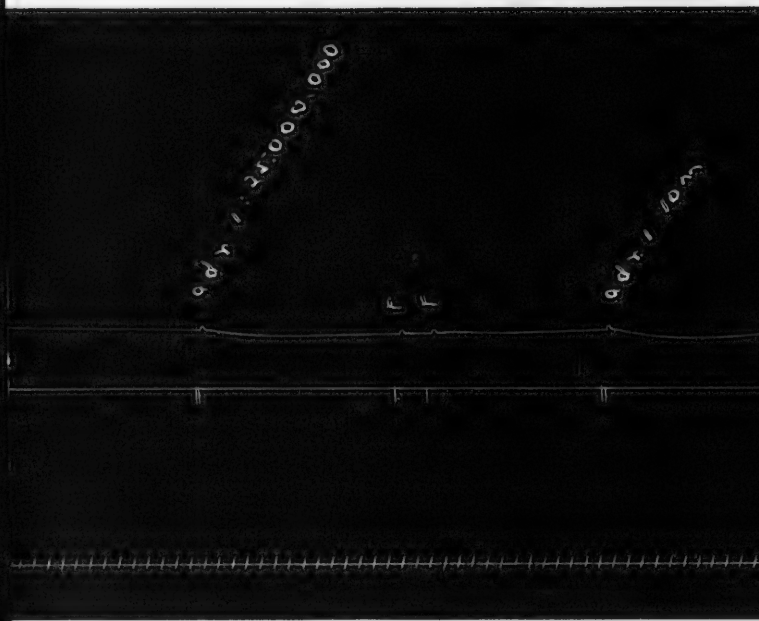
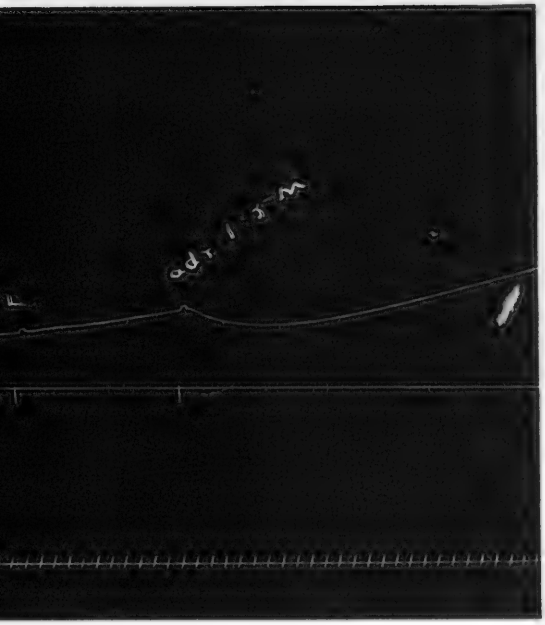


FIG. 1.

PLATE 64.



THE QUESTION OF EPINEPHRIN IN THE CIRCULATION AND ITS RELATION TO BLOOD PRESSURE.*

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The discovery in 1894, by Oliver and Schäfer, of the remarkable rise in blood pressure produced by the injection of suprarenal extract was naturally followed by attempts on the part of physiologists, pathologists, and clinicians to correlate the activities of the suprarenal gland with variations in blood pressure. The hypothesis of increased adrenal secretion as the cause of the high blood pressure in chronic nephritis offered a tempting explanation of this hitherto inexplicable phenomenon. This hypothesis, first proposed by Neusser, later defended by a number of French investigators, Vaquez, Aubertin, and especially Josué, on the basis of supposed anatomical changes in the suprarenal found at autopsy, has been put to the test of experimental verification by many investigators during the past five years. Since the first publication of Schur and Wiesel, who claimed to have identified epinephrin in the blood serum of nephritics by the Meltzer-Ehrmann frog pupil reaction, the results of these studies have been wholly contradictory. Schlayer, Fraenkel, Kretschmer, Trendelenburg, and others were unable to get greater vasoconstriction with the blood of patients having high pressure than with blood from normal persons. Notwithstanding this, practically all observers have believed that the substance in blood serum which produces vasoconstriction, contraction of the uterus, etc., must be epinephrin, because the effects observed appeared to be similar in character to those produced by epinephrin. The earlier studies are so completely reviewed in Biedl's book that extended citation is unnecessary.

The work here described was begun more than a year ago. It

* Received for publication, July 3, 1912.

was undertaken in the hope of discovering the cause of the contradictory results hitherto reported. It has been conducted by a new method which is free from the objections that can be raised against methods used heretofore. Since the inception of the work five important articles, two by Stewart, two by O'Connor, and one by Schultz have appeared and have greatly elucidated the subject, so that the portion of our work dealing with the vasoconstrictor substances of normal serum is now merely confirmatory of their results, although we had been led to a similar conclusion prior to the appearance of their publications.

Stewart in his first paper criticized all previous studies concerning the epinephrin content of blood on the ground that the investigators employed biological test objects on which epinephrin produced only one of its effects, and because control experiments were omitted. He demonstrated that many substances may produce contraction of smooth muscle, of the uterus, or a blood-vessel, and that the effect is similar to that produced by epinephrin. Stewart insisted that before it could be assumed that such reactions are due to epinephrin, the substance tested must also be shown to exhibit the inhibitory effect of epinephrin. He suggested the method of Magnus as a proper control.

O'Connor, fulfilling the conditions laid down by Stewart, in parallel experiments made on rabbit intestine, and either the Fraenkel uterus preparation or the Läwen frog perfusion, showed that the substance in blood serum which exerts a vasoconstrictor effect also causes increased tonus and contraction of the intestinal muscle. He proved conclusively that the vasoconstrictor substance of blood serum is not epinephrin. By further experiments he made the important discovery that this constrictor substance is produced during the process of clotting and is not present in blood which has been obtained in an uncoagulated state. He was unable to identify epinephrin in blood from peripheral veins and arteries of rabbits when kept uncoagulated with citrate. In plasma obtained from the blood of the suprarenal vein of rabbits, however, he believed that he identified epinephrin in dilutions between 1 to 1,000,000 and 1 to 5,000,000. In his most recent article, O'Connor states that he has found the epinephrin content of blood from the suprarenal vein greatly diminished after section of the splanchnic nerves.

Schultz, studying another problem, the reaction of smooth muscle to proteins with particular reference to anaphylaxis, corroborated O'Connor's discovery that the vasoconstrictor substance appears in blood only after coagulation.

Stewart in his latest publication has failed to corroborate O'Connor's finding for blood from the suprarenal vein. He obtained epinephrin reactions constantly only when the gland was handled roughly or massaged, and regularly failed to obtain such reactions when precautions against this were taken, even though the splanchnics were stimulated. Stewart also tested on rabbit uterus and intestine the sera of a number of patients with high blood pressure and other pathological conditions, and failed to obtain evidences of epinephrin. In all this work, however, he employed serum, not unclotted blood, and attempted to detect the modifying influence of the epinephrin on the effect produced by the serum constrictor substance. Such identification of epinephrin in serum is entirely possible, as we have proved repeatedly, and can be seen from Stewart's curves; but from our experience we are convinced that only positive results obtained in this way have any value. Stewart's negative conclusions are invalidated by the fact that he worked with serum. Cannon and de la Paz, working with the Magnus intestine method, have clearly demonstrated epinephrin in the blood of the suprarenal vein in cats.

METHODS EMPLOYED IN THESE EXPERIMENTS.

Arteries.—The method used in the experiments here reported is a modification of the Meyer ox carotid strip method. It fulfills the conditions laid down by Stewart and it has, we believe, distinct advantages over that used by him or by previous investigators. Every experiment was performed in duplicate, one portion of the substance being tested with a carotid or mesenteric artery preparation, which is constricted by epinephrin; the other with a preparation of the coronary, which is relaxed by epinephrin. Instead of dividing the strip of artery, as recommended by Meyer, and attaching the parts to the chamber and the lever by ligatures placed around their ends, two rings of artery were cut and ligatured together. When working with the coronary artery, this procedure is essential

because of the smaller size of the vessel. The results obtained are exactly similar to those obtained from a single, divided ring. The lower ring was secured to the floor of the chamber by a ligature and a hook; the upper one, at a point exactly opposite, was connected by a thread or wire with the writing lever at a point behind its fulcrum. The lever used was a special muscle lever of light construction, so arranged that the magnification could be varied by the distance of the attachment of the preparation from its fulcrum. The loads were hung on the long arm of the lever, which wrote upon a revolving drum making one revolution in four hours. The weight of the lever was carefully eliminated by counterbalancing. The time was marked in one minute intervals. Stretching the artery to overcome its initial tonus was carried on for from twenty to thirty minutes; the load was then reduced to the permanent lifting load, and the artery allowed to come to equilibrium before further steps were taken. The cylinders containing each artery preparation were immersed in water baths maintained at a constant temperature of about 38° C., and the fluid bathing them was oxygenated constantly by a stream of oxygen introduced at the bottom of the cylinder. Locke's solution of uniform composition was used during all preliminary steps, and for washing out the fluids tested.

Certain precautions in technique are necessary. It is most important to maintain a uniform temperature, as close as possible to 37° C. Variations in temperature produce marked alterations in the contraction of the vessel. A uniform oxygen flow is equally necessary, as variations in the stream produce striking changes in the tonus of the vessel. Vessels which have been frozen must not be used.

Proper selection of the loads for stretching and for permanent lifting is important. Accuracy in this direction can only be achieved by practice in estimating the size of the segments used. The protocols hereafter given show the loads employed in a series of experiments. An overloading of the peripheral vessel may result in failure to respond to small doses of epinephrin. Underweighting is apt to keep the vessel in unstable equilibrium so that it responds inordinately to such mechanical stimuli as changing the solutions in the chamber, and produces curves difficult of interpreta-

tion. The effects of varying loads upon the coronary have been described in a separate article by one of us (Park). In working with large magnifications, a proper adjustment of the loads is of great significance.

Care must be taken when changing the solutions in the chamber. This was accomplished by syphoning out from the bottom the contents of the chamber, through a tube which perforated its floor, and introducing the fluid to be tested slowly by means of a pipette from the top. This method obviated any mingling of solutions and all unnecessary jarring of the preparation. Each fluid before being introduced into the chamber was brought to the temperature of the solution which it replaced.

We believe that the use of coronary and peripheral artery preparations, as controls one for the other, has the following advantages for the detection of epinephrin, and particularly in the investigation of the problem of hypertension.

1. The test objects employed are parts of the mechanism under investigation.

2. The test objects are highly sensitive; epinephrin can be identified in a dilution of 1 to 50,000,000 in Locke's fluid and 1 to 20,000,000 in blood.

3. The test objects are not affected by the viscosity of the blood as O'Connor found the frog perfusion preparation to be. Hence dilution is unnecessary in the attempt to detect exceedingly small amounts of epinephrin. This fact makes the negative results of these experiments more valuable than those reported previously. O'Connor was obliged to work with blood diluted 1 to 4.

4. Arteries may be kept for at least six days at a temperature just above freezing; they are, therefore, available for tests at any time.

5. The sensitiveness of the artery preparations does not seem to vary materially during experiments lasting for a number of hours, as is the case with the frog perfusion.

Blood.—The technique for obtaining uncoagulated blood from patients was as follows: Where venesection was performed therapeutically, a paraffined cannula was introduced into the vein. The blood was collected in large paraffined tubes which contained the

anti-clotting solution, kept in ice so that the blood was immediately chilled to a temperature near the freezing point. Where large quantities of blood were drawn, ice and salt were used. When venesection was not desirable, it was possible to obtain from forty to eighty cubic centimeters of blood by means of an ordinary blood culture needle, fitted to a specially constructed tube so curved as to hang below the arm when the needle was inserted into the vein. The needle was boiled in oil and the tube coated with a film of sterile oil before using. The non-clotting solutions used were Locke's fluid with sodium citrate, 1 per cent., to which an equal amount of blood was added; and hirudin dissolved in a small amount of Locke's fluid, four milligrams of hirudin being taken for each seven cubic centimeters of blood. The hirudin blood was practically undiluted; the citrated blood was diluted one half.

The most essential part of the technique is the chilling of the blood. In most experiments the test was begun within an hour of the time of drawing the blood. It was impossible to have the artery preparations in the best condition for the experiment earlier without running the risk of their too long exposure to body temperature. In experiments with unclotted blood, all ligatures were coated with vaselin after the method of Carrel.

EXPERIMENTS PERFORMED.

The experiments fall into two groups.

1. *Defibrinated Blood.*—The earlier experiments, those made prior to the appearance of O'Connor's publication, were performed with defibrinated blood or serum. It was obvious at once that the vasoconstrictor substance present was not epinephrin, as the coronary artery was constricted equally with the peripheral vessel. The subsequent experiments with defibrinated blood were made with a view to elaborating a method for the detection of epinephrin in the presence of the serum constrictor substance. We attempted destruction of the epinephrin in the serum by protracted oxygenation under pressure, with shaking, etc., but arrived at no satisfactory method. The results of these attempts at destruction are of some interest. While the bulk of the epinephrin was evidently destroyed quickly by oxygen at body temperature, some of it seemed to per-

sist for an extraordinary length of time, in one experiment for eighteen hours. These results are in contrast to those reported by Emden and von Fürth, and O'Connor. We do not feel, however, that our experiments are conclusive on this point. O'Connor's discovery that the vasoconstrictor substance appears only after coagu-

A

TEXT-FIG. 1*A*. Ox carotid, 2 rings. Loads: stretching, 90 gm.; lifting, 60 gm. 1. Locke's fluid containing hirudin, 10 mg. in 20 c.c., introduced. 2. Ox blood containing hirudin, 10 mg. in 20 c.c. 3. Defibrinated ox blood. 4. About 1 c.c. epinephrin (adrenalin chlorid 1 : 1,000) added to blood in chamber. Magnification, × 20½ (half size reproduction).

lation of the blood opened the way to a more profitable study of blood from patients showing high pressure, and our destruction experiments were discontinued.

In all, we have tracings from fifteen tests made with defibrinated

B

TEXT-FIG. 1*B*. Ox coronary, 2 rings. Loads: stretching, 30 gm.; lifting, 20 gm. All other conditions as in text-figure 1*A*.

ox blood or serum, one with defibrinated rabbit blood, and three with defibrinated human blood. In every experiment, constriction of the coronary was as marked as constriction of the peripheral vessel (text-figure 1, *A* and *B*). We are, therefore, able to confirm completely the conclusions of O'Connor, Stewart, and Schultz, that the vasoconstrictor substance found in blood serum is not epinephrin. We would go further and take exception to O'Connor's statement in his first conclusion that it is an "epinephrin-like" substance. Judged by its action on the coronary artery and on the intestinal muscle, this substance is antagonistic to epinephrin. Further, the contraction curve produced with a peripheral artery preparation differs distinctly from the curve produced by epinephrin, particularly in the length of time during which a high degree of contraction persists. It is similar to the contraction produced by barium chlorid and to contractions which we have observed when a hypotonic solution of sodium chlorid was substituted for the Locke's fluid bathing the vessel.

We are in entire agreement with Schultz that the substance is one which acts directly upon the smooth muscle of the artery and not, as seems to be the case with epinephrin, upon the so called receptive substance or neuromuscular junction. We are unable to offer a hypothesis as to the exact nature of this constrictor substance.

3. *Whole Blood.*—Twelve experiments were performed with uncoagulated, whole blood.

Of these, two experiments were made with ox blood containing hirudin, four milligrams to each seven cubic centimeters. In one, the coronary test failed through technical error, but the carotid showed no vasoconstriction whatever and subsequently responded promptly to epinephrin. The other experiment is conclusive; neither the coronary nor the carotid showed any change on substituting the blood for Locke's fluid; both contracted promptly upon the introduction of the same blood defibrinated, and finally the carotid showed further constriction and the coronary prompt dilatation upon the introduction of epinephrin (text-figure 1, *A* and *B*).

One experiment was made with rabbit blood diluted one half with Locke's fluid containing 1 per cent. of sodium citrate. In this

experiment, again, there was no sign of an effect upon either the carotid or the coronary.

Nine experiments were made with human blood. The full details of these tests are given in the following protocols.

A

TEXT-FIG. 2*A*. Experiment I, series *B*. Carotid tracing. For details see protocol. Magnification, × 15½.

B

TEXT-FIG. 2*B*. Experiment I, series B. Coronary tracing. For details see protocol. Magnification, × 15½.

PROTOCOLS OF EXPERIMENTS WITH HUMAN BLOOD, SERIES B.

In each case the first load given is the preliminary or stretching load, the second the permanent or lifting load. Mag. indicates lever magnification. The epinephrin used was Parke, Davis and Company's adrenalin chlorid.

Experiment 1.—(Text-figure 2, *A* and *B*). May 2, 1912. Young adult male, robust health. Venous blood by needle, diluted with equal parts of Locke's fluid containing sodium citrate, 1 per cent. Loads: carotid 130 gm., 70 gm.; coronary 25 gm., 20 gm. Mag.: × 15½. Steps of experiment: (1) Locke's fluid plus sodium citrate, 0.5 per cent.; no effect. (2) Citrated blood. Carotid, transient constriction; coronary, no effect. (3) Same blood plus epinephrin, 1:1,000,000. Carotid, moderate constriction; coronary, moderate dilatation. (4) Same blood, defibrinated, diluted four times with Locke's fluid and containing epinephrin, 1:1,000,000. Carotid, further constriction; coronary, slight constriction.

Experiment 2.—May 7, 1912. Normal human placental blood, Sloane Maternity Hospital, diluted with equal parts of Locke's fluid containing sodium citrate, 1 per cent. Loads: carotid not recorded, substantially as in experiment 1; coronary (rather small) 25 gm., 15 gm. Mag.: × 15½. Steps of experiment: (1) Locke's fluid plus sodium citrate, 0.5 per cent. Carotid, slight dilatation; coronary, no effect. (2) Citrated blood. Carotid, slight dilatation; coronary, no effect. (3) Epinephrin to make 1:20,000,000 introduced by pipette. Carotid, slight constriction; coronary, slight dilatation. (4) Epinephrin again introduced in same amount. Carotid, slight further constriction; coronary, no clear effect. (5) Defibrinated ox blood plus Locke's fluid containing 1 per cent. citrate, equal parts. Moderate constriction of both arteries. (6) Washing with Locke's fluid. Carotid, slight dilatation; coronary, arrest of constriction. (7) Undiluted defibrinated ox blood. Carotid, arrest of dilatation; coronary, moderate constriction. (8) Epinephrin, 1:1,000, large dose introduced by pipette. Carotid, moderate constriction; coronary, moderate dilatation.

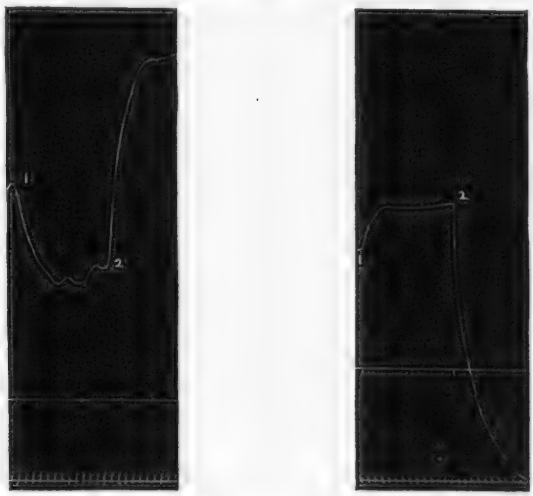
Experiment 3.—May 9, 1912. M.C., female, age 46, Presbyterian Hospital, Ward VII, admitted April 16. Clinical diagnosis: chronic nephritis, cardiac insufficiency. Blood-pressure before bleeding, 210 systolic, 150 diastolic; after bleeding, 240 systolic, 150 diastolic. Blood from vein by needle diluted with equal parts of Locke's fluid containing sodium citrate, 1 per cent. Loads: carotid, 160 gm., 80 gm.; coronary, 35 gm., 20 gm. Mag.: × 15½. Steps of experiment: (1) Locke's fluid containing sodium citrate, 0.5 per cent. No effect on either vessel. (2) Citrated blood. Carotid, no definite effect; coronary, no definite effect. (3) Epinephrin to make 1:30,000,000 by pipette. Carotid, no effect; coronary, very slight dilatation. (4) Epinephrin to make 1:15,000,000. Carotid, no effect; coronary, very slight dilatation. (5) Epinephrin to make 1:1,500,000. Carotid, slight constriction; coronary, moderate dilatation. (6) Washing with Locke's fluid. Carotid, slight dilatation; coronary, moderate constriction with some clotting on vessel. (7) Undiluted, defibrinated blood from this patient. Carotid, no effect; coronary, extreme constriction. (8) As the carotid acted like an overweighted vessel, it was then tested with a large dose of epinephrin. The resulting constriction was slight.

Experiment 4.[1]—May 20, 1912. Patient with nephritis and high tension. Blood from arm vein diluted with equal parts of Locke's fluid containing sodium citrate, 1 per cent. Loads: carotid, 160 gm., 80 gm.; coronary, results invalidated by binding of lever. Mag.: × 41. Steps for carotid: (1) Locke's fluid

[1] This experiment suffers from serious technical errors, probably overweighting.

plus sodium citrate 0.5 per cent.; no effect. (2) Citrated blood; no appreciable change. (3) Washing with citrated Locke's fluid; very slight dilatation. (4) Citrated normal placental blood; slight temporary arrest of dilatation. (5) Citrated placental blood plus epinephrin, 1: 10,000,000; dilatation continued. (6) Washing with citrated Locke's fluid, no effect. (7) Epinephrin in Locke's fluid, 1: 1,000,000; no definite effect. (8) Defibrinated ox blood; slight constriction.

Experiment 5.—(Text-figure 3, *A* and *B*). J. N. H., male, age 39. Presbyterian Hospital, Ward XI, admitted May 17. Clinical diagnosis: chronic ne-



A *B*

TEXT-FIG. 3*A*. Experiment 5, series *B*. Carotid tracing. For details see protocol. Magnification, × 20½ (half size reproduction).

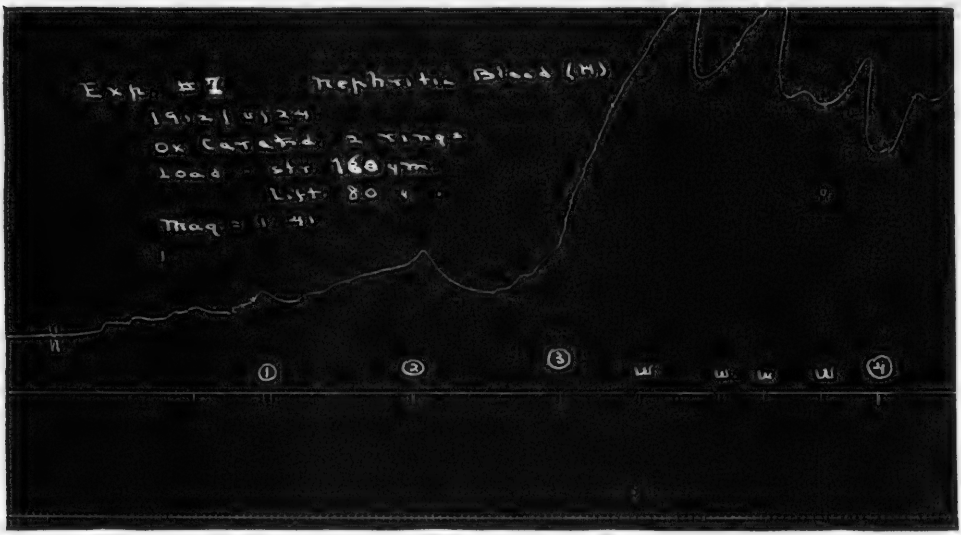
TEXT-FIG. 3*B*. Experiment 5, series *B*. Coronary tracing. For details see protocol. Magnification, × 20½ (half size reproduction).

phritis, cardiac insufficiency, auricular fibrillation. Average blood pressure, 180–190 systolic; 110–120 diastolic. Blood from vein by needle, 20 c.c., diluted with Locke's fluid, 1 c.c., containing hirudin 12 mg. Loads: carotid 140 gm., 80 gm.; coronary, 35 gm., 20 gm. Mag.: × 41. Steps of experiment: (1) Hirudin blood. Carotid, well marked dilatation, then equilibrium; coronary, well marked constriction, then equilibrium. (2) Epinephrin to make 1: 200,000 by pipette. Carotid, extreme constriction; coronary, extreme dilatation.

Experiment 6.—May 24, 1912. E. V., male, age 50, patient of Dr. Victor Agostini. Clinical diagnosis: chronic nephritis, slight cardiac insufficiency, mild uremia. Blood pressure, 200–215 systolic; 150 diastolic. Pulse rate, 100–108. Blood from venesection, with hirudin 4 mg. to each 7 c.c. of blood, dis-

solved in Locke's fluid, about 1 c.c. to 20 c.c. of blood. Loads: carotid, 160 gm., 80 gm.; coronary, 35 gm., 20 gm. Mag.: ×41. Steps of experiment: (1) Cold Locke's fluid. Sharp constriction of both arteries, with rapid dilatation and resumption of equilibrium near former level; coronary, slight subsequent constriction. (2) Locke's fluid at 36° C.; no effect on either vessel. (3) Locke's fluid containing hirudin, 4 mg. to 7 c.c. Carotid, slight temporary dilatation; coronary, slight continued constriction. (4) Hirudin blood. Carotid, slight temporary constriction; coronary, transient constriction followed by marked dilatation, resuming equilibrium. (5) Epinephrin to make 1:180,000, by pipette. Carotid, sharp constriction; coronary, sharp dilatation.

Experiment 7.—(Text-figure 4, *A* and *B*.) Same blood as in experiment 6, after refrigeration for eighteen hours. Loads and magnification as in experiment

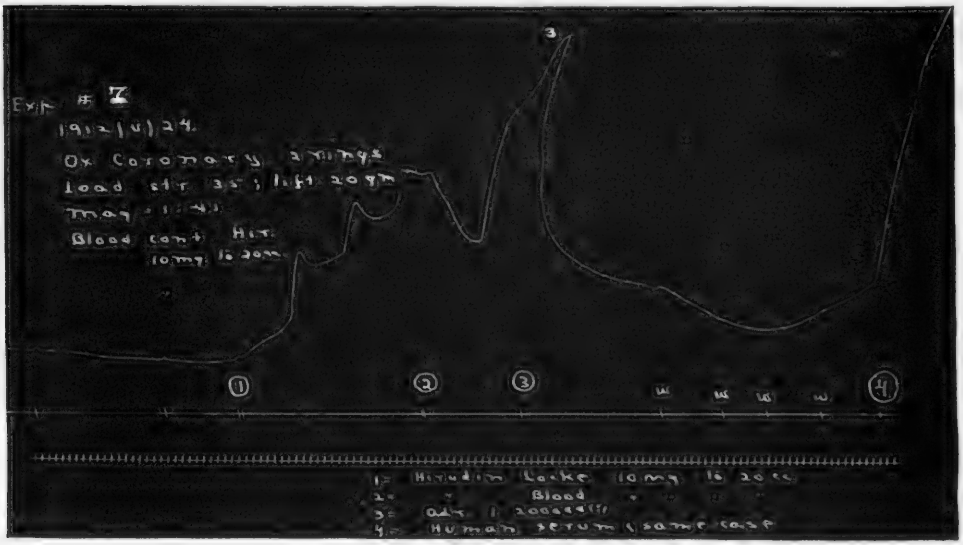


A

TEXT-FIG. 4*A*. Experiment 7, series *B*. Carotid tracing. For details see protocol. Magnification, ×20½ (half size reproduction).

6. Steps of experiment: (1) Locke's fluid containing hirudin. Carotid, transient dilatation, followed by slight continuing constriction; coronary, marked constriction continued. (2) Hirudin blood. Carotid, momentary slight constriction, followed by continuing dilatation; coronary, moderate dilatation, followed by sudden marked constriction. (3) Epinephrin to make 1:200,000. Carotid, extreme constriction; coronary, marked dilatation. W = washing with Locke's fluid. Dilatation of both vessels, with resumption of equilibrium. (4) Defibrinated blood from above case; extreme constriction of both vessels.

Experiment 8.[2]—May 25, 1912. D. S., male, age 64, private patient. Clinical diagnosis: chronic nephritis, moderate polycythemia. Occasional observations during 4½ years. Blood pressure, 170–185 systolic; 95–110 diastolic: before bleeding 170, after bleeding 160. Blood by venesection with hirudin, 10 mg. in 1 c.c. of Locke's fluid to 20 c.c. of blood. Loads: carotid, 150 gm., 80 gm.; coronary, 40 gm., 25 gm. Mag.: ×41. Steps of experiment: (1) Locke's fluid with hirudin, 10 mg. to 20 c.c. Carotid, little effect; coronary, slight dilatation. (2) Blood with hirudin. Carotid, marked dilatation con-



B

TEXT-FIG. 4*B*. Experiment 7, series *B*. Coronary tracing. For details see protocol. Magnification, ×20½ (half size reproduction).

tinuing; coronary, slightly accelerated dilatation. (3) Epinephrin to make 1:18,000,000 by pipette. Carotid, slight check of dilatation; coronary, no clear effect. (4) Epinephrin to make 1:9,000,000. Carotid, further check of dilatation; coronary, no effect. (5) Same blood defibrinated. Carotid, extreme constriction; coronary, no effect.

Experiment 9.—June 8, 1912. H. G., female, age 60, Presbyterian Hospital, Ward VII. Clinical diagnosis: osteophytes of *os calcis*, arterial hypertension without proof of nephritis. Highest pressure, 220 systolic, 110 diastolic; lowest, 190 systolic, 110 diastolic. Blood by venesection with hirudin, 10 mg. in 1 c.c. of Locke's solution to 20 c.c. of blood. Loads: carotid, 160 gm.,

[2] Coronary vessels possibly not living at end of experiment.

90 gm.; coronary, 35 gm., 20 gm. Mag.: ×41. Steps of experiment: (1) Locke's fluid with hirudin, 10 mg. to 20 c.c. Carotid, no definite change; coronary, slight constriction with subsequent equilibrium. (2) Blood with hirudin. Carotid, slight dilatation; coronary, slight dilatation. (3) Blood with hirudin and epinephrin to make 1:20,000,000. Carotid, dilatation checked; coronary, dilatation slightly accelerated. (4) Blood with hirudin and epinephrin to make 1:2,000,000. Carotid, moderate constriction; coronary, moderate dilatation.

From these experiments it is clear that normal human blood preserved from clotting by the addition of sodium citrate, 0.5 per cent., shows no trace of a vasoconstrictor substance. It is also clear that epinephrin added to such blood can be identified readily.

The seven experiments with blood from patients showing high blood pressure represent a study of six cases, five patients with chronic nephritis, and one without evident kidney lesion. In experiment 4 the result is of relatively small value, as the lever attached to the coronary preparation was not working freely. There remain six satisfactory experiments performed with blood from five patients. In none of these is there evidence of a simultaneous epinephrin effect upon both carotid and coronary. At times changes were observed in one artery, which, without the control of the other, might have been interpreted as due to the presence of epinephrin. These changes are difficult to explain. Different bloods have seemed to affect quite markedly the subsequent sensitiveness of the preparations to defibrinated blood and to epinephrin. The substance used to prevent clotting also appeared to have possible toxic effects upon the artery. Sodium citrate in protracted experiments diminished the subsequent response to epinephrin quite definitely. For this reason, and because its use required half dilution of the blood, it was abandoned and hirudin used in all later experiments.

In the first experiment with hirudin blood (experiment 5), a rather striking effect, exactly opposite to that of epinephrin, was produced on each vessel (text-figure 3, *A* and *B*). Upon the subsequent introduction of epinephrin, both vessels gave an unusual response, suggesting that they had been rendered peculiarly sensitive to it. This question of the sensitization of the artery to epinephrin is discussed by one of us (Park) in a separate article dealing with

the coronary. In the six subsequent experiments in which hirudin was used, the arteries were treated with hirudin in Locke's solution before introducing the hirudin blood, in the attempt to eliminate any possible influence of the hirudin itself. Upon such introduction there was no change in the carotid in four instances, and in the coronary in two. There was a slight relaxation of the carotid in two experiments, a slight constriction of the coronary in three experiments, and a moderate constriction in one.

In experiment 8 the substitution of the hirudin blood for Locke's solution containing an identical concentration of hirudin resulted in as striking a relaxation of the carotid as in experiment 5, with the same extreme subsequent response to epinephrin. The coronary effect was negative in this instance, but the coronary preparation was probably overweighted.

It seems probable, therefore, that these effects were due only in small measure to the hirudin itself, and were probably produced by the combination of the hirudin with some protein of the blood, possibly by substances preformed in the blood itself.

CONCLUSIONS.

1. The modified Meyer method here proposed, of parallel tests upon segments of surviving carotid and coronary arteries from the ox, is a satisfactory means for detecting epinephrin in complex body fluids like blood.

2. At the present time there is no evidence that epinephrin, in amounts sufficient to produce its physiological effects upon any hitherto used test objects, exists in the circulating blood, with the exception of blood from the suprarenal vein.

3. The examination of uncoagulated blood from six persons with high blood pressure has failed to show the presence of epinephrin or other constricting substances.

4. The constrictor substance in defibrinated blood and serum is not an epinephrin-like substance. In its point of action and its effects it is similar to barium chlorid. It is a direct stimulant to smooth muscle and seems to have no relation to the sympathetic innervation of muscle.

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THE PHYSIOLOGICAL ACTION OF EPINEPHRIN ON THE BRONCHI.*

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I.

The earlier experimenters (1) in their work on the physiology of the bronchial muscles directed their attention to the effects of stimulation of the vagus. In most instances they used excised, but still living, preparations. They recorded the resulting changes in intrapulmonary pressure generally by means of a water manometer connected with the trachea. Their experiments, dating back to 1840, indicated with considerable uniformity that the result of vagus stimulation is constriction of the bronchi. While their experiments are valuable as a whole, the methods used are open to criticism.

Einthoven (2) was the first to make accurate use of a new procedure, "air perfusion," which has been the basis of the best subsequent experimentation in this direction. By rhythmically injecting air into the trachea of a living animal, he maintained a constant artificial respiration, and was able to determine variations in the size of the bronchi before and after stimulation of the vagus, through records of the changes in the maximal (inspiratory) intratracheal pressure. He found that stimulation of this nerve produced constriction of the bronchi, but he obtained no evidence of any relaxing effect. As section of the vagi produced no change in his results, he concluded that normally the bronchial muscles are not in a condition of vagus tone.

The first experiments concerning the effect of epinephrin on the bronchial muscles were conducted by Dixon and Brodie (3), though it should be remarked that their experiments with epinephrin represented only a small part of a larger work. They used living animals.

* These observations were conducted under the Maria McLean Proudfit Fellowship in Medicine. Received for publication, July 3, 1912.

Their method consisted in measuring variations in volume of the lobe of lung under examination by means of an oncometer, while artificial respiration was kept at a constant. Since no other variable, except possibly the pulmonary circulation, was present (any error due to this they took particular pains to exclude), they attributed the oncometric variation in the inspiratory and expiratory volumes of the lobe to changes in the size of the bronchi. They found that, although stimulation of the vagus produced bronchial constriction, stimulation of the vagus caused inhibition of the spasm, when spasm of the bronchi had been induced through pilocarpin or muscarin. This effect they interpreted as due to the presence of dilator fibers in the vagus, and they were able to demonstrate the presence of such fibers by several degeneration experiments. They got no effect on stimulation of the sympathetic (ganglion stellatum). With regard to the action of epinephrin they write:

> "Suprarenal extract produces very little effect on the bronchi, small tests giving rise to neither constriction nor dilatation. With large injections, however, constriction of a temporary character has been observed in a small percentage of cases. It is possible that in this case the free movements of the air are interfered with by the great vascular engorgement which is present."

When, therefore, epinephrin was used, the possibility of error from changes in the pulmonary circulation could not be excluded; and if any effect at all on the bronchi was produced, that effect was constriction.

Kahn (4) in 1907 had studied the action of epinephrin on the trachea, and had learned that it exerts usually a relaxing effect. But Januschke and Pollak (5) were the first to publish a careful investigation of the physiological action of epinephrin on the bronchial muscles. Employing Dixon and Brodie's method they found that epinephrin caused a slight dilatation of the bronchi. When, however, constriction had been induced by pilocarpin, muscarin, or Witte's peptone, they observed that epinephrin caused a dilatation sufficiently pronounced to offset the former constriction. This dilatation was transient, but could be re-induced by repeating the injection of epinephrin. The dilatation from epinephrin they interpreted as proof of the sympathetic innervation of the bronchi, and suggested, in order to harmonize their view with the findings of Dixon

and Brodie of dilator fibers passing to the bronchi in the vagus trunk, that the latter had acquired sympathetic fibers in the abundant interanastamoses in neck and thorax. It might be remarked that Januschke and Pollak found it necessary to go to some length to prove that their results could not have been modified by changes in the pulmonary circulation.

The most recent publication on this subject is that of Pal (6). He produced spasm in the bronchi of guinea pigs by the use of Witte's peptone or pilocarpin. Certain drugs, as he had previously determined by experiments on other species, relax the coronary artery. He found that these same drugs injected into the guinea pigs caused inhibition of the bronchial spasm. Therefore he inferred that the bronchial and coronary musculatures were physiologically similar and were alike relaxed by epinephrin.

The experiments here reported are based on the reaction to epinephrin of excised rings of bronchi from the ox. The method is the application to the bronchi of that used by Meyer (7) for the study of the reaction of blood-vessels to epinephrin, and by von Langendorff (8) and others to the study of the coronary artery. It is a direct method for the determination of the reaction of the bronchial muscles to epinephrin. Rings of bronchi, two to three millimeters thick, were hung in series in a glass chamber; the lowest ring was fastened to the floor of the chamber, and the upper to the short arm of a lever. The long arm of this lever recorded on a drum. Constriction of these bronchial rings produced an ascent of the curve, dilatation a fall. The glass chamber containing the bronchi was kept filled with Locke's fluid at a temperature of 37° or 38° C. A constant stream of oxygen was passed through the fluid. The mode of introduction of the epinephrin was either to drop it from a pipette or to substitute the desired concentration of epinephrin in Locke's fluid. Medium sized, as well as very small bronchi were used, some so small as to require a needle to thread them. The larger bronchi were hung in pairs, the smaller in series of four, so as to increase the magnitude of the resulting curve. The epinephrin used was an especially prepared "adrenalin" of Parke, Davis and Company, free from preservatives. The maximal concentration was one in fifty thousand, the minimal one in ten million.

Epinephrin caused relaxation of the bronchi in each experiment. As might be supposed, the relaxation was more extensive when medium sized bronchi were used than when very small bronchi were tested. The degree of relaxation was found to vary also with the concentration of the epinephrin. Threshold values, one to twenty million, produced a just distinguishable relaxation of the bronchi. There was no evidence of primary constriction from exceedingly dilute solutions of epinephrin, analogous to the primary constriction which Brodie and Cullis have reported as occurring in their perfusion experiments on the coronary artery. In order to exclude error

TEXT-FIG. 1. Ox bronchus, 2 rings, lumen 2 to 3 mm. Loads: stretching, 10 gm.; lifting, 6 gm. in Locke's fluid. A = epinephrin (adrenalin chlorid) 1:50,000. W = washing with Locke's fluid; B = barium chlorid 2 $M/10$. Magnification, $\times 7\frac{1}{4}$ (half size reproduction).

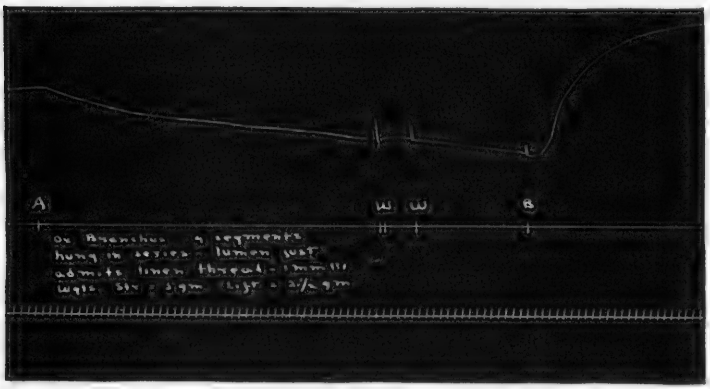
which might be attributable to fatigue or death of the preparations, a known constrictor of living smooth muscle, barium chlorid, was substituted for the epinephrin solution, when the effect of the latter had reached its maximum. Barium chlorid produced a constriction which exceeded the relaxation previously found with epinephrin. The character of the dilatation curve is the abrupt fall which characterizes that of the ox coronary. With the bronchus, as with the coronary artery, it is useless to attempt to compute the extent of relaxation in terms of the diameter or circumference of the bron-

chus used, because this relaxation can be made to vary in the same preparation by a number of different factors (text-figures 1, 2, 3, and 4).

II.

As clearly as the empirical use of a drug in a given disease can indicate its physiological action in that disease, so the use of epinephrin in bronchial asthma has indicated that its physiological action is dilatation of the bronchi. Kaplan in America was the first to direct attention to the clinical fact that epinephrin inhibits asthmatic attacks.

In their exposition of the antagonistic relation between the

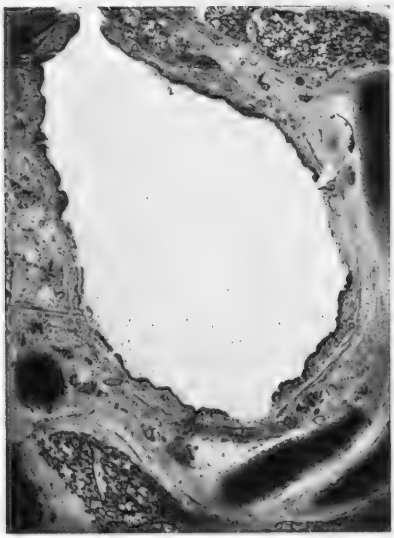


TEXT-FIG. 2. Ox bronchus, 2 rings, lumen just admitted thread. Loads: stretching, 5 gm.; lifting, 2½ gm. in Locke's fluid. A = epinephrin (adrenalin chlorid) 1:50,000. W = washing with Locke's fluid; B = barium chlorid $2M/10$. Magnification, $\times 7¼$ (half size reproduction).

"sympathetic" and "autonomic" (vagus) systems, Eppinger and Hess (9) class bronchial asthma in their group of *vagotonische* diseases. They make the observation, which is corroborated by Frank (10), that in bronchial asthma epinephrin fails to give much, if any, rise in blood pressure. They use this fact to illustrate their statement that epinephrin, related as it is to the sympathetic system, is ineffective in diseases in which the influence of the autonomic system (vagus) seems to predominate. Frank, however, has

demonstrated that epinephrin may cause hyperglycemia in diseases of a *vagotonische* as well as a *sympathicotonische* nature, indiscriminately; in fact, three of his best examples of hyperglycemia occurred in cases of bronchial asthma. By his experimental proof that hyperglycemia induced by epinephrin could occur outside of the *sympathicotonische* state, he deprived the Eppinger and Hess theory of one of its main supports.

Experiments performed by T. C. Janeway and the writer on surviving arteries by the excised strip method would indicate that the possible effect of epinephrin on the excised artery in a physiolog-

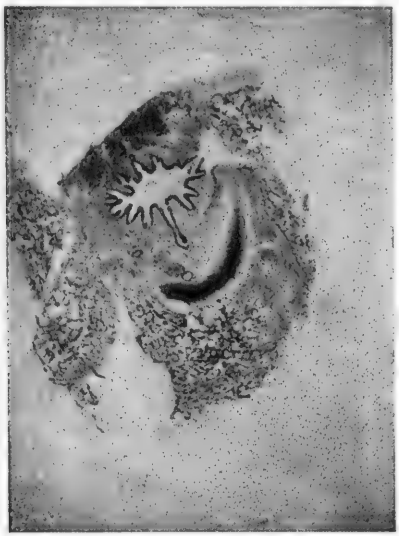


TEXT-FIG. 3. Section of the bronchus used to obtain the tracing shown in text-figure 1. Magnification, × 11¼ (half size reproduction).

ically inert solution is in inverse ratio to the degree of tonus possessed by that artery. By this method a peripheral artery already contracted will not be made to undergo great further contraction from epinephrin, nor will a much relaxed coronary artery show considerable further relaxation. On the other hand, epinephrin will give rise to an unusually great contraction in a much relaxed periph-

eral artery, and a correspondingly great relaxation in a constricted coronary. If the tonus of an artery, then, is optimal for the specific action of epinephrin, the response of that artery to epinephrin will be maximal.

Perhaps, then, it is not so remarkable that in bronchial asthma,



TEXT-FIG. 4. Section of the bronchus used to obtain the tracing shown in text-figure 2. Magnification, × 11¼ (half size reproduction).

an affection in which the bronchial musculature is presumably in a highly constricted state, this relaxing effect of epinephrin on the bronchi should be entirely out of proportion to its effect elsewhere. Indeed, it would seem probable that the effect of epinephrin on the bronchi in bronchial asthma must be due to the very fact that this disease, in so far as the bronchi are concerned, is *vagotonisch* in nature. This view is entirely in accord with those experimental findings of Januschke and Pollak which showed the relaxing action of epinephrin on the bronchi after artificially induced spasm, but it is diametrically opposed to the theory of Eppinger and Hess.

Other instances than bronchial asthma can be cited of the almost

selective action of epinephrin. In certain disturbances of the vascular system characterized by low blood pressure and relaxation of the arterial wall, for instance, in Addison's disease or in shock, the rise in blood pressure following an injection of epinephrin may be enormous. In certain forms of urticaria also, as Swann (11) has determined, an injection of epinephrin causes disappearance of the wheals in a comparatively short period. In both these instances the remarkable effect of epinephrin may be explained on the common ground of the existence of a state of tonus in the involved parts of the vascular system optimal for the action of epinephrin. In the conditions of generally deranged tonus above referred to, the effect of an injection of epinephrin is a general one and manifests itself by a maximal rise in blood pressure, since the entire arterial system, or at least the larger part of it, is in a state of tonus optimal for the action of epinephrin. In urticaria, since only isolated areas of the vascular system, namely, those situated in the wheals, are in a state of optimal tonus, the maximal effect of epinephrin is a local one and manifests itself by disappearance of the wheals, owing to the constriction in them of the arterioles and very possibly the venules also. Surely, in these instances the action of epinephrin on the vascular system, which may be thought of as being in a sensitized state, completely overshadows its action on the bronchial musculature or its action in the production of hyperglycemia; or in the case of urticaria, on the vascular system as a whole. It would be interesting to know whether epinephrin could be regarded as exhausting itself on those body mechanisms which are in a state particularly receptive or optimal for it.

The direct method for determining the reaction of the bronchial musculature to epinephrin used in these experiments is obviously superior to the method employed by Roy and Brown (12) to determine the reaction of the bronchi to stimulation of the vagus and other conditions. Their method consisted in measuring volume changes by means of a thin walled bag introduced into the interior of the bronchus. In several respects, this excised ring method offers advantages over the indirect air perfusion methods which have been described. For example, it obviates all sources of error due to vascular changes in the lungs, which should be considerable when

epinephrin is injected intravenously into a living animal. It avoids also the possibility of error due to swelling of the mucosa of the bronchi; and lastly, since excised structures were used, the action of epinephrin must be exerted directly on the bronchi, that is, on the receptive substances of their musculatures, though there is, of course, no reason to suppose that the law governing the action of epinephrin elsewhere should have an exception here.

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THE CULTIVATION OF MALARIAL PLASMODIA (PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM) IN VITRO.*

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INTRODUCTION.

In November, 1911, one of us (Bass (1)) reported the successful cultivation of *Plasmodium vivax, Plasmodium malariæ,* and *Plasmodium falciparum in vitro.* Though the technique was not given in detail, it was stated that the incubation temperature must be 40° C. or higher, and that defibrinated or citrated human blood seemed to be the most favorable culture medium. Strict anaerobiosis seemed also to be necessary. Further experimentation along this line was interrupted by unavoidable circumstances until April, 1912, when the Tulane School of Tropical Medicine and Hygiene of the Medical Department of Tulane University equipped and sent a research party to the Canal Zone for the study of malaria.[1]

Our efforts were directed toward a critical experimental study of the factors involved in the cultivation of malarial plasmodia *in vitro,* with the hope of developing a technique that would yield more uniform results than had been obtained in the earlier work. The work has resulted in developing a technique by which at least one cycle of schizogony can be cultivated in practically every instance, and many if not an indefinite number of generations can be cultivated in most instances.

* These experiments comprise Study 31 of a series conducted under the direction of Dr. Creighton Wellman. Preliminary Report of the First Research Expedition to the Tropics from the Tulane School of Tropical Medicine and Hygiene. Received for publication, July 19, 1912.

[1] The expenses of this party were paid out of a fund contributed to the School for this purpose through the Dean of the Medical Department of Tulane University, by a person or persons whose name or names are unknown to us. Transportation for ourselves and apparatus was contributed by the United Fruit Company.

Though our prime object was the cultivation of malarial plasmodia we have observed other important facts relative to different phases of the subject of malaria. This communication deals only with the cultivation of malarial plasmodia and is not intended to be a full report of the work done and the observations made by this Commission.

HISTORICAL NOTES.

Laveran (2) reported malarial parasites to be visible in hanging drop preparations for as long a period as ten days. He was apparently not able to satisfy himself that any multiplication took place, nor, indeed, that the parasites remained alive. Coronado (3) claimed to have cultivated malarial plasmodia from infected water, but his work has not been confirmed. In the light of our experiments his claim is at once set aside by the fact that the parasite cannot live for any length of time in any non-isotonic fluid. Such fluids as water are absolutely destructive not only to the red blood cells which are necessary for the growth of the parasites, but also to the plasmodia themselves. Miller (4) reported also that he cultivated the parasites, but his experiments, which have been repeated, have not been confirmed. Sakharov, Rosenbach, Blumer, Hamburger, and Mitchel (5) kept malarial plasmodia alive for several days in leeches that had been allowed to draw the blood of malarial patients. In the instances of Hamburger's experiments, the æstivo-autumnal parasites developed motile pigment. In Blumer's case the parasites showed no ameboid movement. In both instances the parasites became peculiarly refractive and glistening in appearance. Our observation has led us to consider these forms as dead parasites. None of the beautiful segmentation and rosette formation, which are seen in all our successful cultures in forty-eight hours or less, were noted even in eight days. Their cultures, however successful, must be considered as having been accomplished *in vivo* instead of *in vitro*.

We fully appreciate the fact that our present technique is imperfect and includes steps which are more or less empirical. No doubt the present methods will appear very crude a few months hence. They will serve, however, as a basis for further development. Simple as the present technique is, months of patient work and innumerable experiments have been required for its development.

TECHNIQUE.

Apparatus and Material.—The apparatus and material necessary for the cultivation of one generation only of parasites are as follows:

1. Syringe and needle. We use a twenty cubic centimeter all glass syringe with coarse needle. The latter is necessary to avoid strong capillary force and strong suction which so alter the host cells (or parasites), that the parasites often die in a short time. The large needle is also desirable to avoid leaking and bubbling of air into the syringe and to facilitate taking blood into and expelling it from the syringe.

2. Defibrinating tube. Tubes one inch in diameter and of whatever length will be accommodated by the centrifuge (if a centrifuge is to be used) are appropriate. The tube is plugged with a cotton plug having a glass rod running through it and extending to the bottom. We have used with equal satisfaction a glass tube in place of the rod. To this is attached the needle by means of a short piece of rubber tubing. The blood is collected directly into the defibrinating tube by sticking the needle into an infected vein.

3. Culture tubes one half by five inches.

4. Graduated pipette, one cubic centimeter, graduated in hundredths.

5. Dextrose (Merck's), 50 per cent. solution in water.

6. Capillary pipettes and rubber bulb. Three to four sixteenths of an inch glass tubing is the proper size from which to make these pipettes. The capillary tube should be rather large. Fine capillaries are destructive to malarial plasmodia. The large end of these pipettes should be plugged with cotton before sterilizing.

7. Incubator regulated to a temperature of 40° C.

The following additional apparatus will be required if more than one generation of parasites is to be grown, or if it is desired to avoid the dead parasites which are present in the deeper layers of cultures containing a thick column of cells.

8. Centrifuge. Speed, 800 to 2,000 revolutions per minute.

9. Culture tubes one half by five inches, with flat bottom or with disk of pure white filter paper supported one half inch from the bottom of the tube by a piece of glass tubing. The latter should be

cut straight across and fairly smooth on the upper end and should fit the culture tube closely.

10. Plain pipettes, capacity five to twenty cubic centimeters, with rubber tube two feet long, mouth piece, and pinch cock. These pipettes are plugged before sterilizing and are used but once on account of the difficulty of cleaning them.

Technique for Cultivating One Generation Only.—Blood is collected from the patient's vein at the bend of the elbow. If drawn with the syringe it is expelled directly into the defibrinating tube. The latter should be tilted to one side and care should be taken to avoid unnecessary exposure of the blood to the air. In either case, one tenth of a cubic centimeter of the 50 per cent. solution of dextrose for each ten cubic centimeters of blood to be taken is placed in the defibrinating tube before the blood is drawn. Defibrination is effected by gently stirring or whipping with the rod or tube which extends through the cotton plug. The whipping in of air, causing bubbles, must be avoided. The plug and rod may now be replaced by a plug from another tube of the same size.

The defibrinated dextrose blood may be transferred to other tubes or incubated in the original tube. In any event the column of blood must be one to two inches deep. This gives a column of serum one half to one inch deep above the cells and parasites when the latter have settled. Supernatant serum more than one inch deep has no advantage. When this is less than one half of an inch deep the parasites often die before segmentation occurs. We have occasionally seen perfect segmentation, however, under one fourth of an inch of serum.

The parasites live and develop at the top of the column of precipitated cells in a layer varying in thickness from one fiftieth to one twentieth of an inch. All parasites beneath this layer die in from two to twenty hours; the time is dependent upon factors which we have not determined. Some grow considerably before they die and we have occasionally seen small rings attain over half the full adult size under these circumstances. When parasites die in these deep cells the central clear space in the small ring closes, or if the parasite is older the irregular projections, pseudopodia, are withdrawn. If pigment is present, it loses its motility. The protoplasm

gradually shrinks, losing its staining reaction, and finally only the nuclear chromatin granule remains, and this also stains poorly. In the instance of very small parasites often the red blood cell shows little or no evidence of the previous presence of the parasite.

The parasites in the thin layer at the top of the column of cells develop and may be examined at any time by drawing a small quantity of cells from this layer by means of a capillary pipette. Some considerable practice is required in order to do this without drawing cells and dead parasites from just below this layer. The pipette may be passed through a flame to sterilize it on the outside, but it must be allowed to cool thoroughly before it is used, since, in our experience, a temperature of 45° to 50° C. kills the parasites in a very short time.

Such a temperature also alters the red blood cells in some way so that they are rendered permeable to the surrounding serum, and this effect is destructive to the parasites. Great care must be taken in handling tubes containing cultures to keep them in the upright position. Tilting to the side results in burying and killing the living parasites in the thin layer at the top of the cells which have settled to the bottom of the tube.

Technique for Cultivating More than One Generation.—If more than one generation of plasmodia is to be cultivated, it is necessary to remove the leucocytes when the culture is made in order to avoid destruction of the parasites by them at the time of segmentation. The infected blood from the patient is centrifugalized sufficiently to force the leucocytes to the surface of the cells. The length of time necessary to centrifugalize varies with the speed, length of arm, etc., of the particular centrifuge used and should be determined by experiment. Unnecessary centrifugalization should be avoided.

The supernatant serum is drawn off and put in culture tubes. The column of serum in each should be one half to one inch deep. Cells and plasmodia are carefully drawn from about the middle of the centrifugalized cells and planted at the bottom of the serum in the culture tubes. Flat bottom tubes are an advantage. One to two tenths of a cubic centimeter of cells in a half inch tube make the thickest layer in which it is possible to get a homogeneous growth of parasites. We have been able to secure growth of all the parasites

in about twice this quantity of cells in a half inch tube by employing tubes with a paper shelf suspended in them. They are filled with serum to at least half an inch above the level of the support for the paper disk. The disk is then carefully tucked in place, after which the cells are placed on it.

We have obtained even more satisfactory results with tubes filled one half inch deep, or more, with freshly prepared human plasma. These are conveniently prepared by placing one half to one inch of blood, immediately after it is drawn, in culture tubes and centrifugalizing to throw the cells to the bottom before coagulation takes place. By continuing centrifugalization until after coagulation occurs, a flatter surface is secured for the plasma than if it is discontinued before this time. In such plasma prepared tubes one half inch or more of serum is placed and then cells and parasites from the centrifugalized and defibrinated dextrose blood are carefully distributed over the surface of the plasma. We have sometimes obtained in these tubes a layer of live parasites approximately one tenth of an inch thick.

Parasites in such leucocyte-free cultures develop, segment, and most of the merozoites enter new red blood cells. These young parasites develop in the same manner as the first generation and sometimes reach the stage of segmentation. In fact we have in one instance observed the development of three successive generations in such a culture. More often, however, the parasites begin to die out after the first segmentation and especially after the second. We have not been able to determine exactly the cause of this. In order to perpetuate the culture it is necessary to transfer a portion of the cells and parasites to a recently prepared tube containing fresh cells and serum. It is convenient to place the fresh serum in the culture tube and to take up in a large capillary pipette a portion of the cells and parasites of the culture and then about five times the amount of fresh cells. These are mixed in the pipette with air excluded and then carefully spread on the surface of the plasma, paper shelf, or bottom of the tube, according to the particular kind of culture tube used. The transplantation should be done within four or five hours of the time of maximum segmentation and therefore approximately every forty-eight hours for tertian and æstivo-autumnal parasites.

DISCUSSION.

In successful cultures the asexual parasites grow, segment, and form rosettes, which burst and give rise to merozoites, many of which enter new red blood cells in exactly the same manner as they do in the body of man. The young parasites increase in size slowly during the first twenty-four hours, after which they grow much more rapidly. Segmentation begins in about thirty-six hours from the smallest ring-form stage. The chromatin granule first divides into two parts, and in segmenting cultures parasites containing any number up to about twenty segments can be found. The largest number is seen in tertian plasmodia.

Most of our experiments have been with the first and second generations. We have carried one culture of æstivo-autumnal parasites through four generations by the method given above and believe that with sufficient care they can be carried through an indefinite number of generations.

We have cultivated one or more generations of æstivo-autumnal parasites from each of twenty-nine different patients. We have also cultivated the tertian parasite six times. Only one case of quartan was tested, but the parasites failed to grow, as we now believe, on account of certain errors in our earlier technique. It is to be remembered that one of us (Bass (1)) obtained growth of the quartan parasite in his earlier work.

Æstivo-autumnal parasites seem to be more resistant to unfavorable conditions in the cultures and to slight errors in the technique than the tertian. When everything is exactly right, however, the latter grow equally as well as the other variety.

In cultures containing gametes (tertian) we have noticed that the enveloping red blood cell is more resistant to unfavorable conditions and retains its color for a much longer time than cells containing schizonts. Many of these parasites increase in size, and they usually live several hours or days after the asexual parasites have died out. We have not made any systematic study of the sexual parasites, but in many instances we observed what we interpreted as an example of parthenogenesis, and in one instance what we believe was a four day old zygote. These observations indicate the possibility of cultivation of the sexual cycle.

Serum from different individuals is not equally serviceable for the cultivation of malarial plasmodia. The parasites failed to grow in the presence of some of the sera we experimented with. We have cultivated both tertian and æstivo-autumnal plasmodia in the presence of Locke's fluid, minus calcium chlorid, and in the presence of different ascitic fluids, of which one had been drawn nearly two months. Dextrose was always required and the growth was never so good as when human serum was used.

The parasites have been grown in the presence of red blood cells only. We have not seen any evidence that they can be grown independently of these cells. As already stated, they cannot live for even a few minutes free in the serum. Serum inactivated of its complement is less destructive, but the plasmodia cannot live in it for any considerable length of time. When cultures are prepared according to the technique described for the cultivation of one generation only, the leucocytes migrate more or less toward the surface and soon become actually concentrated in the layer in which the parasites grow. Leucocytes do not phagocytize parasites as long as they are inside of the red blood cells, but as soon as segmentation takes place and the capsule of the red cells ruptures, liberating merozoites, the latter are promptly engulfed. In fact, it is not uncommon to see a leucocyte that has phagocytized one or more full rosettes. This probably occurs after the parasite has digested the capsule or otherwise made a small opening through it, whereby it is converted into a foreign body which the leucocytes try to remove. Dead parasites in red blood cells are also phagocytized, providing the enveloping cell substance is sufficiently permeable. Parasites phagocytized are soon killed and finally digested. As a result of this phagocytosis few if any parasites in such a culture escape to develop a second generation.

Other carbohydrates which we tried are saccharose, lactose, galactose, dextrin, mannite, and maltose. Maltose alone seems to be equal to dextrose for this purpose. We are quite certain that the dextrose exercises other important influences than simply the control of oxygen. Blood taken and kept under the most perfect anaerobic conditions will not serve for cultivation of parasites unless dextrose or maltose is added.

We have noted also that blood drawn within one or two hours

after the patient had eaten a full meal was much more serviceable for culture media than if drawn after long fasting. In fact we failed to grow tertian parasites in blood after the patient had been fasting, even though the usual quantity of dextrose was added. This fact is in keeping with the clinical observation that the parasites will often disappear from the blood and the paroxysms cease if the patient is put to bed and is given a purgative and light diet. It is possible that violent exertion and chilling of the surface, both of which often precipitate a malarial chill in patients who would otherwise have missed the paroxysm, act by increasing the dextrose circulating in the blood to meet the requirements of combustion for the production of energy or heat. Or it may be that the dextrose, by reason of its very large molecule, influences the permeability of the red blood cell to substances in the serum which are very destructive to malarial plasmodia. An asexual malarial parasite regardless of its size or age is killed by exposure to normal serum, malarial patient's serum, or any modification of these that we have been able to make. This is particularly noticeable at the time of segmentation of a crop of parasites. If at this time the growing parasites are shaken up or mixed with serum it will be found that even within a few minutes all free merozoites and rosettes whose red blood capsules have become permeable to the serum have died and stain faintly.

The most rapid growth is obtained at a temperature of 40° to 41° C. In fact, æstivo-autumnal parasites will develop from the smallest rings and segment in about thirty hours at a temperature of 41° C., while they require forty-eight hours in the body of man. Parasites will usually develop, but more slowly, at any temperature between 37° and 40° C. We secured one culture of tertian parasites that segmented in four days at the room temperature, in May, of our laboratory at Ancon.

It is highly probable that other red blood cell protozoal parasites of man and animals can be cultivated *in vitro* by this technique or some modification of it. It is also probable that the same principles may be equally applicable to the cultivation of other cell parasites of man and animals. From our observations of malarial plasmodia growing *in vitro* we believe that *in vivo* they can pass from cell to

cell only when a cell is in direct contact with another cell containing a segmenting parasite and then only when the opening for the exit of merozoites occurs opposite the cell to be infected. The substance of malarial plasmodia is very different in consistency from that of red blood cells and therefore they cannot pass through the smallest capillaries like the more yielding fluid-like red blood cell. That the consistency of the protoplasm of the parasite is less yielding than that of the red blood cell is shown by the fact that when a small quantity of a culture containing large parasites is spread over a slide with the end of another slide the parasites are dragged to the end of the spread, though the red blood cells are left behind. Large æstivo-autumnal plasmodia are round or oval; the tertian variety are more or less flattened. If a proper amount of culture containing æstivo-autumnal parasites is placed on a slide and covered with a cover glass, it is found upon studying the specimen under the microscope, while variable pressure is made on the cover glass, that the parasites are not moved, though the red blood cells flow back and forth with great ease. This is due to their round or oval form and firm consistency. Marchiafava and Bignami (6) noted this phenomenon in splenic puncture and autopsy material containing large parasites.

As a result of their unyielding consistency, malarial plasmodia lodge in the capillaries of the body, especially where the current is weakest. Here they remain and develop until they segment. In the meantime other red blood cells are forced against them and if the opening in the infected cell occurs in a favorable location one or more merozoites pass directly into the other cell. Whenever the segmented parasite has become sufficiently broken up it can pass on through the capillary into the circulation where the remaining merozoites are almost instantly destroyed. The debris and pigment are phagocytized by the mononuclear cells of the blood and the endothelial cells of the vessels, and finally are either destroyed or stored in the spleen and other tissues of the body.

We have observed that calcium salts when added to cultures of æstivo-autumnal plasmodia *in vitro* cause hemolysis of the infected red blood cells and possibly also of many non-infected cells. These salts have not the same effect on normal blood. This seems to result

from precipitation of other substances from the serum. The amount of calcium necessary to produce hemolysis is only slightly in excess of the amount present in normal blood and might very possibly be reached by the ingestion of considerable quantities of calcium in drinking water or food. We have herein a suggestion of an explanation of malarial hemoglobinuria. Individual and local susceptibility may be thus explained. What is probably even more striking in this connection is the disappearance of hemoglobinuria, independently of the disappearance of malaria, from localities in which a change from surface lime-containing water to a deep lime-free water supply has been effected.

It is suggested that quinine has no destructive effect upon malarial plasmodia, its effect being possibly to render the red blood cell protecting the parasite more permeable to the all sufficient destructive influence of the serum. If this is true, quinine would affect only the parasites in the circulation and not those lodged in capillaries, which would not be reached by it until they segment. The effect of quinine is defeated by influences such as diet, exertion, etc., which increase the dextrose content of the blood, whereby the permeability of the red blood cells seems to be decreased. These suggestions may possibly lead to a better understanding of the principles involved in the treatment of malaria, and enable us to exchange empiricism for rationalism.

We may have herein an explanation of the manner in which all blood cell parasites of man and animals pass from cell to cell; and it is even possible that the same principles apply to all cell parasites of man and animals.

The size of the capillaries and the amount of blood pressure have an important influence upon the stage at which malarial plasmodia recede from the peripheral circulation. They may be important factors in the production of "cerebral blocking."

SUMMARY.

The asexual cycle of *Plasmodium vivax* and *Plasmodium falciparum* has been cultivated *in vitro* in human blood. The parasites have been grown also in red blood cells in the presence of Locke's solution, free of calcium chlorid and in the presence of ascitic fluid.

The parasites grow within red blood cells and there is no evidence that they can be grown outside of these cells.

The parasites are destroyed in a very few minutes *in vitro* by normal human serum or by all modifications of serum that we have tested. This fact, together with numerous observations of parasites in all stages of growth apparently within red cells, renders untenable the idea of extracorpuscular development.

Leucocytes phagocytize and destroy malarial plasmodia growing *in vitro* only when the parasites escape from their red blood cell capsule or when the latter is perforated or becomes permeable.

Successive generations of *Plasmodium vivax* and *Plasmodium falciparum* have been cultivated *in vitro* by removing the leucocytes from the culture and by transplanting to fresh red blood cells and serum at proper intervals.

The asexual cycle of *Plasmodium vivax* and *Plasmodium falciparum* cultivated *in vitro* does not differ from the same cycle growing *in vivo*.

The sexual cycle has not been cultivated, though we have obtained some evidence of the possibility of its accomplishment.

There can no longer be any doubt that *Plasmodium vivax* and *Plasmodium falciparum* are separate and distinct species. When grown in an identical culture medium and under exactly the same conditions they remain distinct.

In twenty-nine cultures of æstivo-autumnal parasites many forms and sizes have been observed, so that evidence is supplied of the occurrence of different varieties of æstivo-autumnal malarial plasmodia. The so called tertian æstivo-autumnal variety may be seen at the proper stage in all cultures grown from merozoites.

The form and appearance of the same culture of plasmodia may vary greatly under different conditions which are not necessarily destructive to the parasites. Their generation period may vary from thirty hours (æstivo-autumnal) to four days (tertian), as a result of variation in the temperature at which they were cultivated. Sexual parasites grow in the cultures and are more resistant to unfavorable conditions than schizonts, often living several days after the latter die out.

Forms suggesting parthenogenesis have been observed.

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